Rescue of Human Lymphoid Cells from the Effects of Methotrexate in Vitro

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ABSTRACT

In protection experiments (5-formyltetrahydrofolate or 5-methyltetrahydrofolate added to cultures in vitro at the same time as methotrexate), 5-methyltetrahydrofolate afforded the same degree of protection as did formyltetrahydrofolate for phytohemagglutinin-stimulated peripheral blood lymphocytes and for methotrexate-resistant (R) and methotrexate-sensitive (S) sublines of CCRF-CEM. Either reduced folate at 100 µM protected phytohemagglutinin-stimulated lymphocytes or CCRF-CEM-S against 1 µM methotrexate almost completely, against 10 µM methotrexate partially, but not against 100 µM methotrexate. For CCRF-CEM-R 100 µM reduced folate partially protected against 10 µM methotrexate but did not protect against 100 µM methotrexate.

In rescue experiments, cells were exposed in vitro to methotrexate for a 1-hr period, washed, and then resuspended in conditioned medium containing the rescue agent. Again 5-methyltetrahydrofolate equaled 5-formyltetrahydrofolate in efficacy. Either reduced folate at 5 µM rescued phytohemagglutinin-stimulated lymphocytes or CCRF-CEM-S from 50 µM methotrexate, and 500 µM concentrations of either agent rescued CCRF-CEM-S from 50 µM methotrexate, CCRF-CEM-R is not rescued from 500 µM methotrexate (1 hr) by 500 µM agent.

The in vivo rescue of cells after high-dose methotrexate administration is more adequately modeled in vitro by rescue experiments than by protection experiments.

INTRODUCTION

Halpern et al. (15) reported that in vitro cultures of cells of normal origin are protected from methotrexate by the simultaneous presence of either 5-formyl-THF3 or 5-methyl-THF. In contrast, cells of neoplastic origin although protected by formyl-THF were not protected by methyl-THF. On the basis of their results, they proposed that 5-methyl-THF rather than 5-formyl-THF should be administered as the rescue agent after high-dose methotrexate therapy, since only the normal cells that have sufficient methyltransferase activity should be rescued from the toxic effects of the methotrexate.

However, these results are difficult to reconcile with studies by other investigators. Both methyl-THF and formyl-

3 The abbreviations used are: 5-formyl-THF, 5-formyltetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; RPMI 1640, Roswell Park Memorial Institute medium 1640; PHA, phytohemagglutinin.

MATERIALS AND METHODS

5-Formyl-THF was obtained from Grand Island Biological Co., Grand Island, N. Y. (as the calcium salt) and from Lederle Laboratories, Pearl River, N. Y., and 5-methyl-THF (racemic barium salt) was from Sigma Chemical Co., St. Louis, Mo. RPMI medium 1640 was purchased as a dry powder from Pacific Biologicals, Berkeley, Calif., and kanamycin (×100 concentrate) was from International Scientific Industries, Inc., Cary, Ill. Other materials were as previously described (16).

Peripheral Blood Lymphocyte Cultures. Preparation of PHA-stimulated lymphocytes and blast counts of such cultures were performed as previously described (16), with the exception that for certain experiments larger volumes of the lymphocyte suspensions (up to 60 ml) were incubated in 250-ml Falcon plastic flasks (75-sq cm growth area).

Continuous Culture of Established Human Lymphoblastic Cells. The CCRF-CEM cell line previously used (16) was originally cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal calf serum but was later adapted to growth in medium supplemented with 10% human pooled plasma. Surviving cultures of this line subsequently became the resistant subline, or R line, of lymphoblastic cells.

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Another line of CCRF-CEM cells, obtained from Dr. Richard Maca, Department of Internal Medicine, University of Iowa, Iowa City, Iowa has been maintained in medium supplemented with 10% heat-inactivated fetal calf serum and is designated the sensitive subline, or the S line, of lymphoblastic cells. This line has the same sensitivity to methotrexate as that originally determined for CCRF-CEM in this laboratory (16).

It made no difference to the growth or methotrexate sensitivity of either subline whether the medium was supplemented with heat-inactivated fetal calf serum or human pooled plasma. Apart from the question of the medium supplement and the presence in the medium of kanamycin (500 µg/ml), CCRF-CEM lymphoblasts were cultured as previously described (16). The fraction of viable (dye excluding; Ref. 21) cells normally ranged from 85 to 95%.

Protection Experiments with Lymphoid Cells. Experiments in which a tetrahydrofolate derivative and methotrexate were present in the culture throughout the experimental period are designated "protection" experiments. Solutions of methotrexate, 5-formyl-THF, and 5-methyl-THF in RPMI medium 1640 were prepared immediately before use, and concentrations were checked spectrophotometrically from reported molar extinctions (2). The freshly prepared solutions were sterilized by passage through sterile 0.22-µm Millipore filters and added to the lymphocyte cultures in small volumes. In the case of PHA-stimulated lymphocytes, addition was made immediately after the addition of PHA. Blast counts were performed on triplicate cultures after various periods of incubation.

In the case of CCRF-CEM cultures of either subline, cells were harvested from culture by centrifugation and resuspended to a density of 1.5 x 10⁶ cells/ml in fresh medium containing methotrexate and either 5-formyl-THF or 5-methyl-THF at the appropriate concentration and cultured in triplicate in 50 ml (25-sq cm growth area) Falcon plastic flasks. Cell numbers and viability (dye exclusion) were determined at various intervals. After 48 hr incubation unless otherwise noted, only cultures with cell densities of greater than 2.0 x 10⁶ cells/ml were diluted to a density of 1.5 x 10⁶ cells/ml with fresh medium containing the same concentration of methotrexate and 5-methyl-THF or 5-formyl-THF as in the original medium.

Rescue Experiments with Lymphoid Cells. Experiments in which cells were incubated with methotrexate for a limited period of time and were then washed and resuspended in fresh medium containing 5-formyl-THF or 5-methyl-THF but no methotrexate are designated "rescue" experiments.

For rescue experiments with peripheral blood lymphocytes, cultures (50 to 60 ml) were incubated for an appropriate period after PHA stimulation (usually 68 hr). Methotrexate was then added in a small volume of RPMI 1640. After further incubation at 37°, lymphocytes from 15 ml of suspension were harvested by centrifugation at 48 x g (8 min; room temperature) and washed with 15 ml of warm (37°) RPMI medium 1640 containing 20% pooled human plasma. The lymphocytes were centrifuged again and resuspended in 15 ml of lymphokine-containing medium (supernatant medium from identical cultures not treated with methotrexate) that had been supplemented with either 5-formyl-THF or 5-methyl-THF. Sodium ascorbate (50 µg/ml) was also present in the resuspension medium for all cultures to protect the tetrahydrofolate derivatives from oxidation. One-ml portions of the cell suspensions were dispensed into 16 x 100-mm sterile, disposable culture tubes and incubated for a further period. Some comparison cultures were not treated with methotrexate but were washed and resuspended in lymphokine medium containing sodium ascorbate.

In rescue of CCRF-CEM cultures, cells were harvested by centrifugation at 48 x g (8 min; room temperature), and the cells were resuspended to a density of 1.5 x 10⁶/ml with fresh RPMI medium 1640 plus the appropriate serum or plasma supplement. After 24 hr methotrexate was added, and incubation of the cultures was continued for an appropriate period of time. The cells were again recovered by centrifugation, resuspended as before but at 37°, recentrifuged and resuspended in "conditioned" medium (from identical cultures not treated with methotrexate) to which sodium ascorbate (50 µg/ml) and 5-formyl- or 5-methyl-THF had been added. Comparison cultures (not exposed to methotrexate) were similarly treated. The resuspended cells were cultured and counted as in the protection experiments.

RESULTS

Growth of CCRF-CEM Sublines and Sensitivity to Methotrexate. As mentioned in "Materials and Methods," 2 sublines of CCRF-CEM lymphoblasts were used in this study. These sublines exhibit differences in their rate of growth and in their sensitivity to methotrexate. It may be seen from Chart 1 that the growth of subline R is unaffected by the continuous presence of 1 µM methotrexate, whereas the growth of subline S is abolished. Neither the growth nor the inhibition of either subline is affected by the type of medium supplement.

Protection Experiments

PHA-stimulated Peripheral Blood Lymphocytes. Continuous exposure of PHA-stimulated lymphocytes to 50 nm methotrexate prevents the blastogenic response to PHA (16). Formation of blasts, mitosis, and incorporation of deoxuryridine into DNA are all virtually abolished. The protection afforded by 5-formyl-THF to lymphocytes continuously exposed to 1 µM methotrexate is illustrated in Chart 2. Although equimolar amounts of 5-formyl-THF offer little protection from the methotrexate inhibition, increased protection is seen at 10 times this concentration of 5-formyl-THF, and complete protection from the methotrexate (except for the sixth day) is seen with a 100-fold excess of 5-formyl-THF. In 10 µM methotrexate, little protection is afforded by 1, 10, or 100 µM 5-formyl-THF (blast density less than 11, 26, or 57%, respectively, of control after 96 hr, less than 2, 9, or 24% of control after 144 hr), and 100 µM formyl-THF affords no protection against 100 µM methotrexate (data not shown). 5-Methyl-THF was able to protect these cells just as effectively as was 5-formyl-THF (Chart 3) provided that the protecting agent and the methotrexate were added together on the third day after PHA stimulation.
Rescue of Lymphoid Cells from Methotrexate

Chart 1. Effect on the growth of CCRF-CEM sublines of methotrexate and of different serum supplements. O, • results with the methotrexate-resistant strain; □, □, methotrexate-sensitive strain. In A, the medium was supplemented with fetal calf serum and in B, with pooled human plasma. O, □, presence of 1 μM methotrexate; •, □, absence of methotrexate. Bars, S.D. Where none is shown the deviation is smaller than the diameter of the symbol. Arrows, addition of fresh medium. Subsequent cell counts were corrected for this dilution. Total cell, number of viable (dye-excluding) cells/flask.

when the blast count was starting to rise. If, however, these agents were added on the same day as the PHA, 5-formyl-THF was protective (Chart 2), but 5-methyl-THF was not (result not shown). We interpret the inability of 5-methyl-THF to protect under these circumstances as due to its oxidative degradation in the first 3 days of culture before blastogenesis is under way.

This interpretation was substantiated by spectrophotometric observations of the reduced folates under conditions similar to those used in the cell culture experiments. The UV spectra of 5-methyl-THF and 5-formyl-THF solutions (21 and 19 μM, respectively) in a phosphate-bicarbonate buffer identical with that in the medium were recorded at 0, 24, 48, and 72 hr after the solutions were placed in the incubator. While the absorbance of formyl-THF at the maximum (285 nm; Ref. 2) changed less than 5% over the 72-hr period, that of methyl-THF (290 nm; Ref. 2) decreased by more than 40%. After 72 hr, the spectra obtained at pH 1 and 13 were not characteristic of either 5-methyl-THF or 5,6-dihydro-5-methylfolate (11), an observation suggesting that not only was methyl-THF oxidized to the dihydro compound but that further degradation occurred under these conditions.

CCRF-CEM Sublines. Cell growth of subline R is significantly inhibited by 10 μM methotrexate, and each of the tetrahydrofolate derivatives at concentrations up to 100 μM can only partially reverse this inhibition (Table 1). Neither 100 μM formyl-THF nor 100 μM methyl-THF can offer any protection from the growth-inhibiting effects of 100 μM methotrexate (data not shown).

The methotrexate-sensitive subline (subline S) was not protected from 1 μM methotrexate by either of the tetrahydrofolate derivatives when the latter were present at 10 μM concentration, but at 100 μM both offered good protection (Table 1). At higher methotrexate (10 μM), much less protection was afforded by 100 μM methyl-THF or formyl-THF (Table 1), and they afforded no protection against 100 μM methotrexate (results not shown). In all cases methyl-THF gave results very similar to those with formyl-THF.

Rescue Experiments

PHA-stimulated Lymphocytes. The blastogenic response is completely abolished when human peripheral blood lymphocytes (68 hr after PHA stimulation) are exposed to 50 μM methotrexate for 6 hr, and this inhibition is not reversed.
Chart 3. Comparison of the ability of methyl-THF and formyl-THF to protect PHA-stimulated peripheral blood lymphocytes from the effects of methotrexate. On Day 3 after PHA stimulation, the following additions were made to the cultures: •¿. none; O, 1 µM methotrexate; □, 1 µM methotrexate plus 100 µM 5-formyl-THF; ▪, 1 µM methotrexate plus 100 µM 5-methyl-THF.

by the removal of methotrexate and supplementation with 500 µM 5-formyl-THF or 5-methyl-THF (data not shown). A 1-hr exposure to 50 µM methotrexate also abolishes the response to PHA, but incubation in the presence of 5 µM 5-formyl-THF or 5-methyl-THF after removal of methotrexate overcomes the growth inhibition and rescues the cells (Chart 4). Less effective rescue from the effects of 50 µM methotrexate was provided by 0.5 µM methyl-THF or formyl-THF (Table 2). The effects of exposure to 0.5 µM methotrexate for 1 hr can be reversed simply by removal of methotrexate from the medium (data not shown).

CCRF-CEM Lymphoblasts. Exposure of the resistant subline (subline R) of CCRF-CEM to 50 µM methotrexate for 1 hr produces little inhibition of cell growth, although its continuous presence does lead to significant inhibition (Chart 5a). Exposure of subline R to 500 µM methotrexate for 1 hr does produce significant inhibition of growth, but subsequent incubation in the presence of 500 µM 5-formyl-THF or 5-methyl-THF overcomes this inhibition no better than does the partial reversal obtained by simple removal of the drug (Chart 5b).

Exposure of subline S of CCRF-CEM to 50 µM methotrexate for 1 hr produces considerable inhibition of cell growth, which can be largely overcome by the resuspension of the cells in the presence of 5 µM formyl-THF or methyl-THF (Table 2). When the S subline is exposed to 500 µM methotrexate for 1 hr, the growth-inhibitory effects can be partly reversed by either methyl-THF or formyl-THF (Table 2). The reversal is somewhat greater with 50 than with 5 µM rescue agent and is complete with 500 µM (Chart 6).

**DISCUSSION**

This work has demonstrated that human lymphoid cells of normal or neoplastic origin in culture can utilize 5-formyl-THF and 5-methyl-THF to the same extent for the reversal of the inhibitory effects of relatively high concentrations of methotrexate. These results are in contrast to those of Halpern et al. (15) who found that 5-formyl-THF reversed *in vitro* methotrexate inhibition in both the normal cells and neoplastic cells they studied but that 5-methyl-THF reversed inhibition only in normal cells. Our results do not support the proposal (15) that administration of 5-methyl-THF after high-dose methotrexate therapy would be superior to rescue with 5-formyl-THF. The discrepancy between our results and those of Halpern et al. may be partly explained by the relatively low methotrexate concentration (0.04 µM) used by the latter group, but other factors may also be
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Table 1
Protection of cultures of human lymphoid cells by 5-formyl-THF or 5-methyl-THF from the growth-inhibiting effects of methotrexate

Protecting agent and methotrexate were continually present from time zero.

<table>
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<th>Cell type</th>
<th>Methotrexate concentration (µM)</th>
<th>Protecting agent</th>
<th>Concentration of protecting agent (µM)</th>
<th>Blast density as % of control at various times after methotrexate addition</th>
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<td>24 hr</td>
<td>48 hr</td>
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<td>CCRF-CEM-R</td>
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<tr>
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<td>5-Methyl-THF</td>
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<td>100</td>
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<td></td>
<td></td>
<td>5-Methyl-THF</td>
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<td>109</td>
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<td>5-Formyl-THF</td>
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<td>79</td>
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</tbody>
</table>

*Viable (dye-excluding) cells.*

Table 2
Rescue of cultures of human lymphoid cells by 5-methyl-THF or 5-formyl-THF from the growth-inhibiting effects of a 1-hr exposure to methotrexate

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Methotrexate concentration (µM)</th>
<th>Rescue agent</th>
<th>Concentration of rescue agent (µM)</th>
<th>Blast density as % of control at various periods after methotrexate addition</th>
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<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>PHA-stimulated lymphocytes</td>
<td>50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
<td>48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25</td>
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<td></td>
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<td>0.5</td>
<td>57</td>
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<td>5-Formyl-THF</td>
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<tr>
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<td>None</td>
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<td>50</td>
<td>75</td>
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</table>

<sup>a</sup> At 68 hr after stimulation with PHA, peripheral blood lymphocytes were exposed to methotrexate for 1 hr.
<sup>b</sup> Total blasts were counted for PHA-stimulated lymphocytes.
<sup>c</sup> At 24 hr after subculturing, CCRF-CEM cells were exposed to methotrexate for 1 hr.
<sup>d</sup> For CCRF-CEM viable (dye-excluding) cells were counted.

Validity of In Vitro Models. In the treatment of neoplastic disease with high-dose methotrexate, serum methotrexate concentrations reach about 10 µM (1, 18, 28) or even into the mM range (23, 25, 27), and these concentrations persist for periods of 4 to 36 hr. Furthermore, rescue is achieved by an amount of 5-formyl-THF 1 to 30% of the methotrexate dose with the lower end of the range being more usual (7, 17, 18, 23, 25, 27, 28). It is therefore probable that the serum concentrations of rescue agent achieved are considerably lower than is the methotrexate concentration reached.

By contrast, in our protection experiments in which the ability of 5-methyl-THF or 5-formyl-THF to reverse the growth-inhibiting effects of methotrexate were studied with inhibitor and rescue agent both continuously present in the...
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Chart 5. Growth-inhibiting effect of methotrexate on CCRF-CEM subline R and its reversal. Cells were subcultured into fresh medium, and 24 hr later methotrexate was added, and incubation continued for 1 hr. The cells were then washed and resuspended in conditioned medium that in some cases contained rescue agent or methotrexate. In A, 50 μM methotrexate was used. Cells were resuspended in conditioned medium alone (■) or in conditioned medium containing 50 μM methotrexate (○). A comparison culture was not treated with methotrexate, but cells were washed and resuspended (●). In B, the comparison culture was not treated with methotrexate (■), but other cultures were treated with 500 μM methotrexate and resuspended in conditioned medium (□) or in conditioned medium containing 500 μM 5-methyl-THF (■), 500 μM 5-formyl-THF (△), or 500 μM methotrexate (○).

culture medium, we found that even high concentrations of rescue agents afforded only partial protection (Table 1) and that very little protection was possible from methotrexate concentrations of above 10 μM. This is in agreement with the results of others who used high concentrations of methotrexate in protection experiments (22, 24). However, Halpern et al. (15) obtained complete reversal of inhibition in protection experiments with 5-formyl-THF or 5-methyl-THF at only twice the concentration of the methotrexate, a result possibly related to the low concentrations of methotrexate used (0.04 to 0.08 μM).

The in vitro requirement for high concentrations of 5-formyl-THF (or 5-methyl-THF) to reverse the effects of high methotrexate concentrations on lymphoid cells in protection experiments may be partly due to competition between formyl-THF and methotrexate for a common membrane transport system such as that demonstrated in both normal (3, 9) and malignant cells (10, 12-14, 20). Such competition would be more pronounced in the in vitro protection experiments than during in vivo rescue after high-dose methotrexate where extracellular methotrexate levels are falling at the time of administration of 5-formyl-THF. In addition, differences in the metabolic effects of methotrexate and the rescue agent in vitro from those in vivo must also be involved. Possibly, the degree of inhibition of dihydrofolate reductase is greater and more prolonged in the in vitro situation, but this has not been demonstrated.

In our in vitro rescue experiments in which cells were exposed to high methotrexate concentrations for 1 hr, washed, and resuspended in conditioned medium containing rescue agent, considerable reversal of growth inhibition of both PHA-stimulated peripheral blood lymphocytes and of the sensitive subline of CCRF-CEM was achieved by rescue agents at only 1% of the methotrexate concentration used (Table 2). Furthermore, rescue could be achieved after exposure to methotrexate concentrations of up to 500 μM. In these in vitro rescue experiments, therefore, the concentration of rescue agent required for reversal of growth inhibition is only 10−4 times that required in protection experiments. Despite the limitations on the information that

Chart 6. Growth inhibition of CCRF-CEM subline S by methotrexate and its reversal by reduced folates. Cells were subcultured into fresh medium, and 24 hr later 500 μM methotrexate was added, and incubation was continued for 1 hr. After washing, the cells were resuspended in conditioned medium (■) or in conditioned medium containing 500 μM 5-methyl-THF (□), 500 μM 5-formyl-THF (△), or 500 μM methotrexate (○). A comparison culture was not treated with methotrexate but was washed and resuspended in conditioned medium (●).
can be gained by this type of in vitro rescue experiment, the model seems worth exploring further.

Behavior of the Resistant Subline of CCRF-CEM. The results with the resistant subline differed considerably from those obtained with PHA-stimulated peripheral blood lymphocytes or with the methotrexate-sensitive line of CCRF-CEM. The resistant line not only required much higher levels of methotrexate to inhibit growth in both protection and rescue experiments, but also reversal of inhibition was only partial even at high levels of formyl-THF in both protection and rescue experiments (Table 1; Chart 5). The resistance of this subline is not due to any increase in the level of dihydrofolate reductase and is presumably due to defective transport of methotrexate and reduced folate derivatives. The results of the protection and rescue experiments are certainly consistent with this explanation. The behavior of this subline in vitro does in fact follow the pattern assumed to explain selective kill of neoplastic cells in vivo by high-dose methotrexate therapy and formyl-THF rescue (8), since the resistant cells are not rescued at concentrations of formyl-THF at which normal cells are.

Competitive Nature of Rescue and Protection. Our findings on the competitive nature of the reversal of methotrexate inhibition by formyl-THF or methyl-THF (Tables 1 and 2) is in agreement with the previous work of Pinedo et al. (22) who presented considerably more data on such a relationship in the protection of bone marrow cells and with that of Borsa and Whitmore (4, 5) who demonstrated that formyl-THF competitively reversed the toxicity of methotrexate for mouse L-cells in tissue culture. Although in these protection experiments (4, 5, 22) and in our own competitive reversal may be explained by a shared transport system, the basis for competitive reversal in rescue experiments in vitro must have a different basis.

REFERENCES

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