Methotrexate Rescue by 5-Methyltetrahydrofolate or 5-Formyltetrahydrofolate in Lymphoblast Cell Lines

Nicholas P. B. Dudman,1 Peter Slowiaczek, and Martin H. N. Tattersall2

Ludwig Institute for Cancer Research, Blackburn Building, University of Sydney, New South Wales 2006, Australia

ABSTRACT

The rescue of lymphocytes from methotrexate (MTX) growth inhibition by 5-methyltetrahydrofolate (5-methyl-THF) and 5-formyltetrahydrofolate (5-formyl-THF) has been studied. Rescue by 5-methyl-THF is selective for cells with high levels of homocysteine:5-methyl-THF methyl-transferase (methyltransferase). At MTX concentrations which inhibited growth ≥85% in both leukemic T-lymphocytes (CCRF-CEM) and Epstein-Barr-transformed B-lymphocytes (LAZ-007), 5 μM 5-formyl-THF rescued more effectively than did 5-methyl-THF, in either the presence or absence of the methyltransferase inhibitor, nitrous oxide. At less inhibitory MTX concentrations, both reduced folates rescued equally, except when methyltransferase was inhibited by nitrous oxide in which case 5-formyl-THF was clearly superior.

In the absence of nitrous oxide, both cell lines contained approximately equal amounts of methyltransferase. Some apparent differences in the rescue of these cell lines with 5-methyl-THF were attributable to their different sensitivity to MTX.

When metabolism of reduced folates was severely impaired by MTX and nitrous oxide, lymphocytes were rescued with 5-[methyl-14C]methyl-THF, and the uptake of 14C into DNA was measured. Incorporation was very low, indicating that cellular oxidation of 5-methyl-THF to 5,10-methylene-tetrahydrofolate is minimal even under forcing conditions.

MTX selectivity in vivo will be influenced by the level of methyltransferase in tumor and normal tissues.

INTRODUCTION

MTX has been in clinical use for more than 30 years as an antitumor agent and as an immunosuppressant drug (6, 7). MTX has a broad spectrum of antitumor activity including leukemia, lymphoma, choriocarcinoma, and breast, head and neck, and bladder cancers (4–7, 15, 20). Following studies in animal tumors by Goldin (8–10), programs utilizing 5-formyl-THF as an antitumor agent and as an immunosuppressant drug (6, 7).

Our understanding of the mechanism by which citrovorum factor rescue has been the subject of much debate and experimental work. It has been suggested that the effects of high-dose MTX are not reversed in tumor cells which lack reduced folate transport mechanisms (4, 7), but there are no data demonstrating impaired reduced folate transport in human tumors (3, 19). Halpern et al. (12) studied reduced folate protection against MTX poisoning in a number of cultured mammalian cell lines. They reported that 5-methyl-THF protected normal but not malignant cells from MTX toxicity, while citrovorum factor showed no selectivity in protection and was equally effective at preventing MTX toxicity in normal and malignant cell lines. Most of the cell lines were examined for their content of the vitamin B12-dependent enzyme methyltransferase (2). It was noted that those cells which were not protected from MTX poisoning by 5-methyl-THF had much lower enzyme levels than those which were protected (12). Ash et al. (2) and Halpern et al. (12) found that cell lines with low methyltransferase activity were in general malignant (with the exception of normal human amnion cells), while normal cell lines had much higher levels of methyltransferase. Because of this finding, they proposed that 5-methyl-THF might be preferred as a rescue agent for MTX treatment in cancer chemotherapy, compared to the more widely used citrovorum factor rescue.

Nixon and Bertino (17) studied citrovorum factor metabolism in humans using tracer amounts of radiolabeled material given p.o. and i.v. They observed that 5-formyl-THF was rapidly anabolized to 5-methyl-THF, but these experiments were performed without preceding MTX treatment.

Grotto and Blakley (11) examined the reduced folate protection and rescue of cultured lymphoblasts from MTX poisoning. In the former case, reduced folates were present in the medium both during and after exposure of the cells to MTX, while in the latter circumstances, reduced folates were added to cell culture medium after MTX exposure. The concentrations of MTX and reduced folates were much greater than those used by Halpern et al. (12), and 5-methyl-THF and 5-formyl-THF were said to be equally effective at similar concentrations in both protection and rescue experiments (11).

At present, our understanding of the mechanism by which citrovorum factor, administered some hr after MTX, may prevent normal tissue toxicity without impairing antitumor effects is incomplete. We have reexamined MTX rescue by 5-methyl-THF and 5-formyl-THF under a variety of conditions using 2 lymphoblast cell lines: the leukemic T-cell line CCRF-CEM and the Epstein-Barr virus-transformed B-cell line LAZ-007. We have looked for differential MTX rescue between the 2 reduced folates and for conditions under which this might occur. Following the report (1) that N2O strongly inhibited methyltransferase, we examined the effects of N2O exposure on cells also exposed to MTX, in the presence of 5-methyl-THF. These experiments led to the investigation of whether, during rescue by 5-methyl-THF, this compound was oxidized by the enzyme 5,10-methylene-THF reductase.

1 Present address: Department of Medicine, Clinical Sciences Building, Prince Henry Hospital, Little Bay, New South Wales 2036, Australia.
2 To whom requests for reprints should be addressed.

Received May 11, 1981; accepted November 5, 1981.

The abbreviations used are: MTX, methotrexate; 5-formyl-THF, 5-formyltetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; methyltransferase, 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13); 5,10-methylene-THF, 5,10-methylene-tetrahydrofolate; RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; SDS, sodium dodecyl sulfate.
MATERIALS AND METHODS

Radioactive compounds were obtained from the Radiochemical Centre (Australia) Pty., Ltd. Sigma Chemical Co., St. Louis, Mo., supplied 5-methyl-THF, DL-homocysteine thiolactone, S-adenosylmethionine, cyanocobalamin, polyvinyl sulfate, and Dowex 1 ion-exchange resin. Calbiochem (Australia) Pty., Ltd., supplied sodium ascorbate and 2-mercaptoethanol. 5-Formyl-THF, donated as calcium leucovorin by Lederle Laboratories, Australia, was converted to the ammonium salt and purified free of methyl and propylparaben by column chromatography on Whatman DEAE 52 (21). All other chemicals were of analytical grade. Gases were bought from Commonwealth Industrial Gases Pty., Ltd., Australia.

Materials for tissue culture media were supplied by Flow Laboratores (Australasia) Pty., Ltd., and tissue culture flasks were from Crown Corning, New York, N. Y.

Radioactive samples were counted after preparing them in aqueous solution (1.0 ml) to which scintillant was added (10 ml). The scintillant contained, in 2 liters, 630 ml Triton X-100, 8 g PPO, 0.2 g POPP, and toluene to volume. Samples were counted in an LKB Ultratak liquid scintillation counting system.

Continuous Culture of Human Lymphoblast Cells. CCRF-CEM and LAZ-007 cells (gifts of Dr. H. Lazarus, Sidney Farber Cancer Center, Boston, Mass.) cells were grown at 37° as suspension cultures in RPMI 1640 supplemented with L-glutamine (2 mm), NaHCO3 (0.1%), and fetal calf serum (10%). The pH of the medium was adjusted to approximately 7.4 with 1 m NaOH. MTX or folates were added as detailed in “Results.” Gassing of cell cultures was achieved by lightly bubbling the cell suspension with gas sterilized by passage through a 0.2-μm Millipore filter. The flasks were gassed until bubbles reached their necks and were immediately sealed. They were regassed each time they were opened for sampling or for additions.

Cell densities were measured by counting samples on a hemocytometer using a phase-contrast microscope, except for the experiment measuring the sensitivity of the cell lines to different concentrations of MTX. In that case, cells were counted with a Celsoscope Model 401 particle counter.

Response of Cells to MTX. Tissue culture flasks (75 sq cm) were inoculated with 100 to 150 ml of a suspension of logarithmically growing cells at 2 × 10^6 cells/ml. Various concentrations of MTX were added and cells were allowed to grow for 3 days (LAZ-007) or 5 days (CCRF-CEM) before being counted.

MTX Retention. In some experiments, lymphoblasts were incubated with MTX for 1 hr before being washed and resuspended in a medium containing various additives. In order to establish the concentration of MTX in the final incubation mixture after this procedure, the following control measurements were carried out. CCRF-CEM and LAZ-007 cells were suspended at 1.5 × 10^6 and 10^6 cells/ml, respectively, in 10-ml aliquots of medium containing 5.4 μM [3^2,5^2,7^-3H]MTX (0.77 Ci/mmol). After 1 hr at 37°, the homogenate was harvested. Their DNA was extracted and purified, and its specific radioactivity was measured using an adaptation of the method of Khy et al. (13) as follows. Silanized glassware was used in all steps, and approximately 5 × 10^5 cells were extracted per treatment. Cells were centrifuged (200 × g, 10 min) and washed twice with Dulbecco’s phosphate-buffered saline. The cell pellet was then resuspended and homogenized with 5 ml 0.1 m Tris-HCl buffer, pH 9.0, which contained 1% SDS, 5 mm EDTA, and 10 mg polyvinyl sulfate per liter. The homogenate was incubated at 37° for 30 min and then centrifuged at 12,000 × g for 15 min. The supernatant was extracted with 0.1 volume of phenol reagent (phenol saturated with SDS buffer), the biphase mixture being vortexed frequently during the 15-min incubation. The phases were separated by centrifugation, and the phenol layer was reextracted with SDS buffer (2 ml). This step was repeated, and all aqueous phases were combined and then treated with 0.1 volume of CHCl3:octanol (5:1 by volume) for 10 min with vigorous shaking. The aqueous phase was collected by centrifugation (12,000 × g, 10 min) and treated with NaCl (5.8 mg/ml). DNA and RNA were precipitated by adding 2.5 volumes of ethanol at −20° and allowing the preparation to stand for 30 min at −20°. The precipitated material was collected (12,000 × g, 20 min) and washed once with 70% ethanol at −20°. The white precipitate was dissolved in 4 ml H2O, and the RNA content was hydrolyzed with 0.3 m NaOH (1 hr, 37°). The preparation was neutralized with HCl, and 10% excess was added followed by 0.1 volume of 5 m HClO4. The sample was placed on ice for 15 min and filtered through a 0.22-μm Millipore filter. The filter was washed twice with ice-cold 0.5 m HClO4 and placed in 3.0 ml 0.1 m NH4OH to dissolve the DNA. The preparation was vortexed and centrifuged. The UV spectrum of the supernatant was measured, and the radioactivity of 1- or 2-ml samples of the supernatant was monitored.

RESULTS

Sensitivity of CCRF-CEM and LAZ-007 Cells to MTX

Under the growth conditions used, the MTX concentration which inhibited cell growth by 50% compared with control cultures growing in the absence of MTX for LAZ-007 cells was 5.0 × 10⁻⁸ m and for CCRF-CEM cells 1.4 × 10⁻⁸ m. Table 1 shows the results from the experiment described in “Materials and Methods” in which MTX concentrations were measured in cell suspensions exposed to 5.4 μM [3^2H]MTX and then washed and resuspended in fresh medium. By examination of cells on hemocytometer grids, the diameter of the LAZ-007 lymphoblasts was found to average about one-third larger than that of

Once with 10 ml fresh medium, and then resuspended in a further 10 ml fresh medium containing 5-methyl-THF or 5-formyl-THF (5 μM).

Solutions of folates which were added to cell suspensions as rescue agents had been prepared by dissolving them in RPMI 1640 containing sodium ascorbate (5 mg/ml) or in oxygen-depleted RPMI 1640, which had been bubbled previously with high-purity N2 for 20 min. These folate solutions were sterilized by filtration. Upon addition of folates, cultures were gassed and incubated at 37°.

5-Methyl-THF Uptake. Cells were grown for 24 hr in the presence or absence of N2O. Cultures were then challenged with 50 μM MTX for 1 hr at 37° and then washed and resuspended in fresh medium containing 5 μM 5-[methyl-¹⁴C]5-methyl-THF (58 mCi/mmol), with or without N2O. After further incubation at 37° for 15 to 350 min, cultures were centrifuged (1200 × g, 2 min), and the cell pellet was quickly washed twice with 10 ml ice-cold Dulbecco’s phosphate-buffered saline. The final pellet was dissolved in 1.0 ml H2O and counted for radioactivity.

Incorporation of Methyl-¹⁴C from 5' Methyl-¹⁴C5-methyl-THF in DNA. In some experiments, cells were exposed to N2O for 24 hr and then challenged with MTX for 1 hr. After rescue by 5 μM [methyl-¹⁴C]5-methyl-THF (45.4 mCi/mmol) for 6 to 24 hr, cells were washed and harvested. Their DNA was extracted and purified, and its specific radioactivity was measured using an adaptation of the method of Khym et al. (13) as follows. Silanized glassware was used in all steps, and approximately 5 × 10^5 cells were extracted per treatment. Cells were centrifuged (200 × g, 10 min) and washed twice with Dulbecco’s phosphate-buffered saline. The cell pellet was then resuspended and homogenized with 5 ml 0.1 m Tris-HCl buffer, pH 9.0, which contained 1% SDS, 5 mm EDTA, and 10 mg polyvinyl sulfate per liter. The homogenate was incubated at 37° for 30 min and then centrifuged at 12,000 × g for 15 min. The supernatant was extracted at 65° with 1 volume of phenol reagent (phenol saturated with SDS buffer), the biphase mixture being vortexed frequently during the 15-min incubation. The phases were separated by centrifugation, and the phenol layer was reextracted with SDS buffer (2 ml). This step was repeated, and all aqueous phases were combined and then treated with 0.1 volume of CHCl3:octanol (5:1 by volume) for 10 min with vigorous shaking. The aqueous phase was collected by centrifugation (12,000 × g, 10 min) and treated with NaCl (5.8 mg/ml). DNA and RNA were precipitated by adding 2.5 volumes of ethanol at −20° and allowing the preparation to stand for 30 min at −20°. The precipitated material was collected (12,000 × g, 20 min) and washed once with 70% ethanol at −20°. The white precipitate was dissolved in 4 ml H2O, and the RNA content was hydrolyzed with 0.3 m NaOH (1 hr, 37°). The preparation was neutralized with HCl, and 10% excess was added followed by 0.1 volume of 5 m HClO4. The sample was placed on ice for 15 min and filtered through a 0.22-μm Millipore filter. The filter was washed twice with ice-cold 0.5 m HClO4 and placed in 3.0 ml 0.1 m NH4OH to dissolve the DNA. The preparation was vortexed and centrifuged. The UV spectrum of the supernatant was measured, and the radioactivity of 1- or 2-ml samples of the supernatant was monitored.

RESULTS

Sensitivity of CCRF-CEM and LAZ-007 Cells to MTX

Under the growth conditions used, the MTX concentration which inhibited cell growth by 50% compared with control cultures growing in the absence of MTX for LAZ-007 cells was 5.0 × 10⁻⁸ m and for CCRF-CEM cells 1.4 × 10⁻⁸ m. Table 1 shows the results from the experiment described in “Materials and Methods” in which MTX concentrations were measured in cell suspensions exposed to 5.4 μM [3^2H]MTX and then washed and resuspended in fresh medium. By examination of cells on hemocytometer grids, the diameter of the LAZ-007 lymphoblasts was found to average about one-third larger than that of
**Table 1**

MTX retention by cell cultures

<table>
<thead>
<tr>
<th>MTX concentration ($\times 10^{-9}$M)</th>
<th>Radioactivity in cells after 24 hr (dpm/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspensions after washing</td>
<td>Supernatant after 24 hr</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>2.76</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>2.36</td>
</tr>
</tbody>
</table>

the CCRF-CEM cells. This means that, although both cell types retain the same radioactivity after 24 hr, the concentration of labeled MTX and its metabolites is some 2-fold higher in the CCRF-CEM cells than in the LAZ-007 cells. This may be linked to the greater sensitivity of the CCRF-CEM cells towards MTX.

**Toxicity of Ascorbate towards CCRF-CEM Cells.** Based on the work by Groft and Blakley (11), our initial studies utilized 0.25 mM ascorbate when reduced folates were added to the medium in order to maintain the added folates in their reduced form. However, ascorbate was found to inhibit growth in both cell lines and was omitted from all subsequent experiments. Similar toxicity of ascorbate has been reported previously (14).

**Effect of N2O on Reduced Folate Rescue of MTX-treated Cells**

**LAZ-007 Cells.** LAZ-007 cells were treated with N2O 24 hr before exposure to 50 $\mu$M MTX for 1 hr. Subsequently, the cells were washed free of external MTX and rescued with either 5 $\mu$M 5-methyl-THF or 5-formyl-THF; N2O was present throughout the rescue period. In the absence of N2O, MTX strongly inhibited growth of these cells; poisoned cells could be rescued with comparable efficacy by both reduced folates although 5-formyl-THF was slightly better (Chart 1A). Although cell growth was slower in the presence of N2O, MTX was still strongly inhibitory and both reduced folates rescued. 5-Methyl-THF was again marginally less efficacious than was 5-formyl-THF (Chart 1B).

**CCRF-CEM Cells.** An experiment in which CCRF-CEM cells were treated in the same way gave somewhat different results. In these cells, inhibition of growth by N2O was negligible, but inhibition by MTX was more marked than in the case of the LAZ-007 cells. In the absence of N2O (Chart 2A), rescue by 5-formyl-THF was clearly superior to rescue by 5-methyl-THF; however, when N2O was present (Chart 2B), rescue by 5-methyl-THF was seriously compromised, but rescue by 5-formyl-THF was not affected.

**Effects of N2O on Methyltransferase Activity and 5-Methyl-THF Transport**

Methyltransferase activity/mg of protein in LAZ-007 cells averaged 81% of that in the CCRF/CEM cells (range, 49 to 160%). The possibility that N2O was inhibiting the methyltransferase in the CCRF/CEM cells but not in the LAZ-007 cells was investigated by assaying the enzyme from cells exposed for 24 hr to N2O (Table 2). In both lines, the activity of the methyltransferase was drastically reduced by exposure to N2O even when the assays contained added vitamin B12 which is considered to be the target for N2O inhibition (1). Exposure of cells to N2 under similar conditions did not reduce methyltransferase activity.

Transport of labeled 5-methyl-THF in both cell lines was studied in the presence and absence of N2O. N2O caused no significant difference in the uptake of label by either cell line.

**5-Methyl-THF Metabolism**

The possibility that LAZ-007 cells might metabolize 5-methyl-THF by a pathway not inhibited by N2O was investigated. One possibility considered was that, in the severe deficiency of reduced folates following MTX treatment, reversal of the en-
zyme-catalyzed reduction of 5,10-methylene-THF might occur. Thus, 5-methyl-THF would be oxidized to 5,10-methylene-THF, which would then participate directly in the methylation of dUMP or would be further oxidized and contribute its one carbon moiety to purine biosynthesis.

Experimentally, if 5-[methyl-14C]methyl-THF were metabolized by demethylation followed by the normal utilization of tetrahydrofolate, very little methyl-14C should be incorporated into DNA (primarily as epigenetically labeled methylcytidine). On the other hand, oxidation to 5,10-[methyl-14C]methylene-THF would result in the incorporation of 14C label into thymine methyl and purine bases at both positions 2 and 8.

It was anticipated that, if 5,10-methylene-THF reductase were catalyzing the dehydrogenation of 5-methyl-THF in LAZ-007 cells but not in the CCRF-CEM cells, then rescue from MTX poisoning by 5-[methyl-14C]methyl-THF should reveal an upswing in the specific radioactivity of LAZ-007 DNA in the presence of N2O but no marked change in the CCRF-CEM cells. A number of experiments were carried out in which cells from both lines were incubated in N2O for 24 hr prior to 50 μM MTX for 1 hr and then washed and incubated in 5 μM 5-[methyl-14C]methyl-THF for either 6, 22, or 24 hr. The cells were then harvested, and the DNA was extracted and purified. The UV spectrum of extracted DNA showed it to be substantially pure; λmax, 257 nm, A280:A260 = 0.51 to 0.54. Results from representative experiments are shown in Table 3.

The specific activity of DNA always increased in MTX-treated cells grown under N2O (Table 3). If the cells were not exposed to MTX, the overall incorporation of 14C into DNA was higher, particularly in the CCRF-CEM cells. However, N2O treatment alone generally lowered 14C specific activity in DNA compared to control cells.

These results indicated that both LAZ-007 and CCRF-CEM cells were capable to a limited extent of metabolizing 5-methyl-THF to 5,10-methylene-THF, then to thymidylate and purines, and finally to DNA. There was no clear-cut difference between the 2 cell lines in their response to N2O.

The possibility that the differential response of the 2 cell lines to rescue from MTX poisoning by reduced folates was linked to their different MTX sensitivities was investigated. Both cell lines were exposed to varying concentrations of MTX for 1 hr after having been incubated in N2O for 24 hr. They were then washed free of external MTX and resuspended in 5 μM 5-methyl-THF or 5-formyl-THF and regassed with N2O. Forty-one hr later, the cells were counted. Under these conditions, MTX inhibited growth in CCRF-CEM cells at about one-tenth of the concentration required for similar inhibition of LAZ-007 cells (Chart 3). In each cell line, 5-methyl-THF and 5-formyl-THF rescued cells with similar efficacy at MTX concentrations which inhibited growth by about one-third. At higher MTX concentrations, 5-methyl-THF was a poorer rescue agent for both cell lines and even 5-formyl-THF did not rescue fully. Chart 4 shows the results of similar experiments conducted in the absence of N2O. In the CCRF-CEM cells, both reduced folates maintained a high level of rescue until a concentration of MTX was reached which reduced growth to less than 15% of control cultures. At higher MTX concentrations, rescue became increasingly ineffective, with rescue by 5-methyl-THF falling more rapidly than 5-formyl-THF. In the LAZ-007 cells, the higher concentration of MTX used inhibited growth by 85%, but there was no clear difference between rescue by 5-methyl-THF and 5-formyl-THF.

In repeat experiments, the growth of LAZ-007 cells was inhibited by more than 85% at the highest MTX concentration and rescue by both reduced folates fell, 5-formyl-THF from 70.5 to 54% and 5-methyl-THF from 67.5 to 42%. The CCRF-CEM cells behaved as described above.

### Sensitivity of CCRF-CEM and LAZ-007 Cells to N2O

N2O inhibited the growth of LAZ-007 cells more than CCRF-CEM cells. Control experiments were carried out by gassing cells with either N2 or N2O and counting the cells daily for 3 days. In each cell line, the growth of cells was affected to the same extent as N2O or N2, with significant inhibition after 72 hr. These results suggest that oxygen depletion above the medium caused by N2O or N2 gassing is responsible for growth inhibition. Apparently, LAZ-007 cells are more sensitive to oxygen depletion under these conditions than are the CCRF-CEM cells.

### DISCUSSION

Our results showed that 5-formyl-THF rescued both CCRF-CEM and LAZ-007 cells from MTX poisoning more effectively than did 5-methyl-THF when growth inhibition by MTX became substantial or when methyltransferase was inhibited by N2O. In the presence of MTX and N2O, both cell lines apparently metabolized the methyl carbon of 5-methyl-THF into DNA without using methyltransferase. The CCRF-CEM and LAZ-007 cells contained approximately equal amounts of methyltransferase; however, the CCRF-CEM cells were more sensitive to MTX growth inhibition than were the LAZ-007 cells.

In comparing the rescue from MTX poisoning by 5-methyl-THF and 5-formyl-THF in vitro, the apparent superiority of 5-formyl-THF might be due to its greater resistance to oxidation, allowing it to persist longer in the medium than 5-methyl-THF. We have shown, however, that 5-methyl-THF supported the growth of both cell lines for approximately 2 days to about the same extent as 5-formyl-THF, provided that growth inhibition by MTX did not exceed 30% under N2O and 85% without N2O. Furthermore, Halpern et al. (12) showed that 5-methyl-THF would support the growth of some cell lines after an otherwise

---

**Table 3**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Exposure to rescue folate (hr)</th>
<th>N2O (A)</th>
<th>No N2O (B)</th>
<th>A:B</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAZ-007</td>
<td>MTX</td>
<td>6</td>
<td>1186</td>
<td>493</td>
<td>2.41</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>MTX</td>
<td>24</td>
<td>4312</td>
<td>3025</td>
<td>1.43</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>No MTX</td>
<td>24</td>
<td>4898</td>
<td>3139</td>
<td>1.56</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>MTX</td>
<td>22</td>
<td>1623</td>
<td>1549</td>
<td>1.09</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>MTX</td>
<td>6</td>
<td>861</td>
<td>634</td>
<td>1.36</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>No MTX</td>
<td>6</td>
<td>2531</td>
<td>3783</td>
<td>0.669</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>MTX</td>
<td>6</td>
<td>134</td>
<td>107</td>
<td>1.25</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>No MTX</td>
<td>6</td>
<td>604</td>
<td>642</td>
<td>0.941</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>MTX</td>
<td>6</td>
<td>379</td>
<td>163</td>
<td>2.32</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>No MTX</td>
<td>6</td>
<td>5883</td>
<td>6516</td>
<td>0.903</td>
</tr>
</tbody>
</table>

* Specific activity equals dpm/A260 unit.

† Both results from a braced pair were obtained concurrently from the same cell preparations and are directly comparable.
N. P. B. Dudman et al.

lethal dose of MTX. This growth lasted several days and was equivalent to the growth support given by 5-formyl-THF. These results suggest that differential susceptibility of the reduced folates to atmospheric oxidation is not an important consideration.

The progressive failure of rescue by 5-methyl-THF as MTX concentration increased suggests that MTX interferes with either the uptake or metabolism of 5-methyl-THF but not with that of 5-formyl-THF. Currently, there are no reports of the effects of MTX treatment on 5-methyl-THF metabolism in vivo.

Halpern et al. (12) advocated the use of 5-methyl-THF in rescue of patients following MTX treatment. This proposition was based primarily on their findings that normal cells have higher levels of methyltransferase than malignant cells. This proposition is examined by our studies with CCRF-CEM cells exposed to MTX under normal circumstances and in the presence of N2O which inhibited the methyltransferase. When these cells are growth inhibited by MTX between 15 to 150 /ÌM in the presence of N2O, 5-formyl-THF is a much more effective rescue agent than 5-methyl-THF, while in the absence of N2O both folates have similar rescue capabilities. This model is consistent with the idea (2, 12) that 5-methyl-THF rescues cells with high methyltransferase more effectively than similar cells with low methyltransferase. The present findings (compare Charts 3 and 4) suggest that cell lines with very low methyltransferase may be more sensitive to MTX poisoning than cells with high methyltransferase.

Groff and Blakley (11) have reported results which apparently argued against the idea that 5-methyl-THF may rescue or protect MTX-treated cells less well than 5-formyl-THF. However, methyltransferase levels in the cell types they studied were probably not low. Our results and previous assays (17) show that CCRF-CEM cells have similar or higher levels of methyltransferase compared with other lymphocytes. The specific activity of methyltransferase in pho- tohemagglutinin-stimulated lymphocytes is at least 3-fold higher than in resting normal lymphocytes. Thus, no differential rescue between 5-methyl-THF and 5-formyl-THF would have been expected in the experiments (11) on the basis of limiting levels of methyltransferase. Table 4 compares our results with those of Groff and Blakley (11). In both sets of data, 5 /ÌM 5-formyl-THF is more effective than is 5-methyl-THF, but at 500 /ÌM MTX rescue by 5-formyl-THF in our experiments is substantially more than in the previous work (11). This difference might be ascribed to variations between the 2 laboratories in their use of conditioned medium or ascorbate or purified 5-formyl-THF. In any case, the work reported by Groff and Blakley (11) does not appear to rule out the possibility of differential rescue by reduced folates.

The idea that lymphocytes metabolized the methyl carbon of 5-methyl-THF directly into DNA (presumably via 5,10-methylene-THF) is suggested by our data since in the presence of N2O cells were at least partially rescued from MTX poisoning by 5-methyl-THF (Chart 3). Furthermore, labeling of DNA by 5-[methyl-14C]methyl-THF in the presence of MTX consistently increased under N2O. It has also been established that purified 5,10-methylene-THF reductase will catalyze the oxidation of 5-methyl-THF to 5,10- methylene-THF (16). However, the small increase in the incorporation of 14C into DNA under N2O in our experiments suggests that the pathway is very minor.

Clinically, much of the 5-formyl-THF administered to patients either p.o. or i.v. is probably rapidly metabolized and appears in the plasma as 5-methyl-THF (17). Methyltransferase required to process this compound is seriously deficient in a number of malignant cell lines compared with normal tissues (11). It is thus possible that some human tumors contain low levels of methyltransferase such that they are not readily rescuable from severe MTX poisoning by 5-methyl-THF. These tumors may also be sensitive to MTX without rescue, because of their inability to utilize the normal plasma folate 5-methyl-THF.

In conclusion, our results show that 5-methyl-THF can be a selective rescue agent of cells poisoned by MTX. Cells which have had their folate metabolism more seriously inhibited by MTX are less readily rescued. Furthermore, cells with high methyltransferase are rescued more completely than those with low methyltransferase. N2O has been used to prepare cells with reduced methyltransferase activity, and the possibility of in vivo modulation of MTX toxicity by N2O should be considered. Evidence has been presented that 5-methyl-THF
can be oxidized to 5,10-methylene-THF by 5,10-methylene-THF reductase but that it is a minor pathway.

REFERENCES

Methotrexate Rescue by 5-Methyltetrahydrofolate or 5-Formyltetrahydrofolate in Lymphoblast Cell Lines

Nicholas P. B. Dudman, Peter Slowiaczek and Martin H. N. Tattersall