Development of an Assay System for the Detection and Classification of Methotrexate Resistance in Fresh Human Leukemic Cells

Sjoerd Rodenhuis, John J. McGuire, Ramaswamy Narayanam, and Joseph R. Bertino

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

An assay system was developed for the detection and classification of methotrexate resistance in fresh human leukemic cells. Mechanisms of resistance to be identified were: overexpression of dihydrofolate reductase, decreased cellular uptake of methotrexate, decreased affinity of dihydrofolate reductase for methotrexate, decreased polyglutamylation of methotrexate, and low thymidylate synthase activity. The initial screening procedure utilizes $^3$H release after addition of [5-$^3$H]-2'-deoxyuridine as a measure of intracellular activity of thymidylate synthase and of DNA synthesis; $^3$H release is assayed after 3-h incubations with methotrexate, trimetrexate, or $\gamma$-fluoromethotrexate and after 4-h incubations with these agents followed by a 6-h incubation in drug free medium. The pattern of DNA synthesis inhibition and recovery under these two sets of conditions establishes the presence or absence of methotrexate resistance and allows a tentative classification of the resistance mechanism involved. In combination with determinations of dihydrofolate reductase activity, methotrexate titration studies, and the determination of intracellular drug accumulations in vitro, the system is readily able to classify CCRF-CEM human leukemia cell lines possessing well defined mechanisms of resistance. The findings in seven leukemia patients are also reported. Applying tentative reference values, four patients showed biochemical evidence of methotrexate resistance: two patients had only a transport defect, one patient had evidence of both a transport defect and low thymidylate synthase activity, and one patient appeared to have decreased methotrexate polyglutamylation. Application of the assay system in larger numbers of patients is feasible and is required to establish adequate reference values for the evaluation of biochemical-clinical correlates.

INTRODUCTION

Drug resistance, natural or acquired, is considered to be the most important reason for treatment failure in patients with cancer (1). The mechanisms whereby malignant cells become resistant have been the subject of an intense research effort in recent years and a large variety of such mechanisms has been identified (for review see Ref. 2). One of the most comprehensively studied drugs in this respect is MTX, and the detailed understanding of mechanisms of resistance against this drug in vitro has led to the proposal of several strategies to overcome resistance by the selective eradication of resistant cells (for review see Refs. 3 and 4).

Four mechanisms of resistance to MTX have been extensively studied in vitro. These include (a) increase of the level of DHFR, the target enzyme of MTX, often as a result of DHFR gene amplification (5–10); (b) decrease of MTX transport into the cell (11–13); (c) alteration of DHFR, resulting in an enzyme with decreased affinity for MTX (14, 15); and (d) decrease of intracellular polyglutamylation of MTX. In contrast to the parent compound, polyglutamylated MTX is retained intracellularly for prolonged periods of time and it is implicated in the cytotoxic mechanism of MTX. MTX polyglutamates bind to DHFR as tightly as MTX itself and additionally inhibit several other enzymes that require folate cofactors (for review see Ref. 16).

A further determinant of resistance to MTX may be the activity of TS. TS catalyzes the reductive methylation of dUMP to dTMP using 5,10-methylenetetrahydrofolate as the cofactor. The other product is dihydrofolate and in the absence of functional DHFR (such as in the presence of MTX) continued TS activity leads to depletion of the intracellular reduced folate pool. Low intracellular TS leads to a decreased rate of loss of reduced folates, which in turn allows a higher degree of inhibition of dihydrofolate reductase to be endured without damage to the cell (17–19).

The relative contributions to clinically encountered drug resistance of the mechanisms outlined above are unknown. Transport defects may occur not infrequently (20). Three patients with clinical MTX resistance associated with DHFR gene amplification have been described (21–23). In each of these instances, gene amplification was of low magnitude (2- to 4-fold). The possible simultaneous presence of a second mechanism of resistance, e.g., a transport defect, was not investigated. DHFR enzymes with significantly decreased affinity for MTX have been reported to be present in myeloblasts of some untreated patients with acute nonlymphocytic leukemia (24), possibly contributing to the natural resistance that is frequently encountered in this disease.

Strategies to specifically eradicate MTX-resistant cells are usually directed at cells with a transport defect (3, 4). Cells with impaired MTX uptake also have an impaired ability to take up reduced folates from the medium and are therefore expected to be preferentially damaged by a state of folate deficiency. Folate deficiency may be induced by a low folate diet (25) or more conveniently by using the folate-cleaving enzyme carboxypeptidase G2 (26). Cells with MTX-transport impairment remain sensitive to the “nonclassical” antifolate TMQ (27), which accumulates to high intracellular levels independent of the reduced folate carrier system (28). Treatment with trimetrexate followed by “rescue” with leucovorin may thus be selectively toxic to the transport-defective cells (26). In contrast, cells with acquired drug resistance due to DHFR-gene amplification are usually cross-resistant to TMQ (29). In this case, produgs that are converted to active cytotoxic compounds by DHFR may be exploited, e.g., homofolate and dihydrohomofolate (30).

Before strategies such as these can be tested in patients, more information is required with respect to the frequency of occurrence of the different types of resistance. In addition, for the selection and monitoring of patients, an assay system is needed that allows the detection of MTX resistance in patient material, and that is also able to identify the type(s) of resistance involved within a short period of time. Based on short-term DNA
synthesis inhibition assays previously reported by us (20), we have developed a test system that satisfies these requirements for sensitive and resistant cultured human lymphoblasts and that was successfully used to study fresh human leukemic cells.

MATERIALS AND METHODS

Chemicals. Trimetrexate-glucuronate was obtained from Warner-Lambert/Parke-Davis, Ann Arbor, MI, and was dissolved in sterile water. Methotrexate was obtained from the National Cancer Institute, Bethesda, MD, and was dissolved in saline. The concentrations of both drugs were determined by absorbance, using a mm extinction coefficient of 242 nm of 41.1 (pH 1) for TMQ and of 22.0 at 257 nm (pH 13) for MTX. 7-Fluoromethotrexate was obtained as gift from Dr. J. K. Coward, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY (31). [5-3H]-2'-Deoxyuridine and [3H]folic acid were purchased from Moravek Biochemicals, City of Industry, CA. Dihydrofolate was synthesized by the method of Blakley (32). [3H]Dihydrofolate was obtained by reduction of [1H]folic acid as described (33). Enzymatically reduced NADPH and PHA were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest purity available.

Media, sera and antibiotics for tissue culture were purchased from Grand Island Biological Co., Grand Island, NY.

Cell Lines. A cloned subline of the human T-lymphoblast cell line CCRF-CEM (34) was used, as well as three MTX-resistant sublines: CCRF-CEM/R1, characterized by a 18-fold DHFR gene amplification (35), CCRF-CEM/R2, which has normal DHFR levels but is MTX resistant due to impaired transport (36), and CCRF-CEM/R3 that displays both DHFR-gene amplification and transport impairment (27).

A fourth resistant cell line, CCRF-CEM/R4, was obtained by infecting the parent CCRF-CEM line with a defective, amphotrophic DHFR retrovirus derived from Abelson murine leukemia virus into which a cDNA coding for an altered DHFR enzyme with reduced affinity for methotrexate was introduced (37). This cell line is cultured in the presence of 10–3 M MTX and expresses the DHFR derived from resistant Swiss 3T6 mouse embryo fibroblasts (38). All cells were grown in RPMI 1640, supplemented with 10% horse serum and had been cultured in drug-free medium for at least 2 weeks prior to each experiment.

Processing of Patient Samples. Leukemic cells were obtained from the peripheral blood or bone marrow of patients with acute leukemia or with blast crisis of chronic myelogenous leukemia. The blast cells were isolated by standard Ficoll-Hypaque density centrifugation and washed twice in complete medium (RPMI 1640 containing 10% d-falayed fetal calf serum). If the total cell yield equalled or exceeded 180 x 10^6 cells, a complete test could be performed. Cells (40 x 10^6) were washed twice in 0.9% NaCl solution and frozen at –20°C for later preparation of DNA and crude extracts. The remaining cells were either used immediately or stored overnight in RPMI 1640 containing 30% fetal bovine serum. Cells in excess of 180 x 10^6 were resuspended in ice-cold RPMI 1640 containing 10% fetal bovine serum and 10% dimethyl sulfoxide and directly placed in a –70°C freezer for later use.

PHA-stimulated Lymphocytes. Peripheral blood mononuclear cells of healthy volunteers were isolated by standard Ficoll-Hypaque density centrifugation of 20 ml of heparinized peripheral blood. The cells were washed twice in α-modified Eagle’s medium and were resuspended (density, 1 x 10^6 cells/ml) in α-modified Eagle’s medium containing 20% fetal bovine serum and 1% (vol/vol) PHA. The cells were then incubated for 36 to 48 h at 37°C in a humidified atmosphere containing 5% CO2. The cells were subsequently washed in RPMI 1640 containing 10% dialyzed fetal bovine serum (complete medium) and resuspended in 4 ml complete medium for the [3H]deoxyuridine assay described below.

Brief Rationale of the Method. Biochemical MTX resistance, in particular as a result of DHFR overexpression, decreased MTX uptake, or decreased affinity of DHFR for MTX, can be detected by assaying deoxyuridine incorporation into DNA after incubation with MTX (39). The pattern of resistance to MTX, TMQ, and FMTX may yield information on the mechanism of resistance involved. MTX resistance due to gene amplification may be associated with resistance to other antifolates as well (TMQ), whereas resistance as a result of decreased transport of MTX will not affect TMQ sensitivity since the latter compound enters the cell through a pathway independent of that of MTX. A defect in polyglutamylation is expected to be characterized by inhibition of DNA synthesis after incubation with MTX, but rapid recovery after incubation in drug-free medium (as a result of the rapid efflux of MTX or MTX with only one additional glutamate). An altered DHFR can be identified by MTX inhibition curves. Increased intracellular DHFR levels will be found in case of DHFR overexpression and gene amplification may be present as determined by DNA dot-blotting. A decreased intracellular accumulation of MTX indicates a transport defect. An increase of the intracellular [TMQ]/[MTX] ratio is a more sensitive measure for this (see below). The abnormalities expected to be found in resistant cells with different mechanisms of resistance are indicated in Table 1.

[5-3H]-2'-Deoxyuridine Tritium Release Assay. The inhibition of DNA synthesis by antifolates was estimated using an assay for the in situ determination of thymidylate synthase activity (40). After cellular uptake of [5-3H]-2'-deoxyuridine and conversion to [5-3H]dUMP, triptidium is released as H2O into the aqueous medium when thymidylate synthase replaces the 5'-group by a methyl group.

The cells were suspended to a density of 10^5 cells/ml (20 x 10^6 cells/ml for fresh patient cells) in complete medium and divided into nine 400-µl aliquots in capped 12- x 75-mm culture tubes. Five of the aliquots were incubated for 3 h in the presence of, respectively, no drug, 1 µM MTX, 0.1 µM MTX, 0.1 µM TMQ, and 5.0 µM FMTX (31). [5-3H]-2'-Deoxyuridine was then added to a final concentration of 1 µCi/ml and 100-µl samples were taken at 0, 15, 30, and 45 min. These were added to 200 µl of a 15% activated charcoal suspension in 4% trichloroacetic acid, mixed, and centrifuged for 5 min at 16,000 x g. One hundred µl of the supernatant were added to 5 ml scintillation fluid and quantitated using a liquid scintillation counter. Four aliquots were incubated for 4 h in the presence of, respectively, no drug, 1 µM MTX, 1 µM TMQ, and 5 µM FMTX. The cells were subsequently washed three times in complete medium that had been prewarmed to 37°C, suspended in drug-free complete medium, and incubated for an additional 6 h. The H3 release test was then performed as described for the 3-h incubation.

The results of the scintillation counts were analyzed by calculating the slopes of the linear 3H release curves using linear regression, and were expressed as a percentage of the slope of untreated control cells.

DHFR Activity Assay. Cells (40 x 10^6) were washed twice in 0.9% NaCl solution and resuspended in 50 mM sodium phosphate buffer, pH 7.0. Crude extracts were obtained by freeze-thawing the cells three times, followed by centrifugation at 16,000 x g for 15 min. The DHFR activity in the supernatant was determined using the radioassay of Hayman et al. (33) with several modifications, as described below. In addition to the appropriate volumes of crude extract, the 0.2-ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM NADPH, 0.2 mg KCl, 0.1 mM [3H]dihydrofolate (3–5 x 10^6 cpm/µmol), 1 mg/ml bovine serum albumin, and 25 µM mercaptoethanol. The reaction was started by the addition of NADPH and allowed to continue for 20 min at 37°C. The reaction was stopped by placing the reaction tubes on ice and by the addition of 25 µl 0.1 M folate. The folate and dihydrofolate were precipitated by the addition of 25 µl 25% acetic acid. No ZnSO4 was added. After leaving the tubes on ice for an additional 10 min, the precipitate was pelletted by a 10-min centrifugation at 16,000 x g and 100 µl of the clear supernatant were counted in a scintillation counter. The background radioactivity level was determined by a control reaction without NADPH and a second control without crude extract.

Methotrexate Titration. To determine the affinity for MTX, the DHFR activity in crude extracts was determined at different concentrations of MTX. For this purpose, the appropriate amounts of bovine serum albumin, KCl, and NADPH were added to the crude extracts (see “DHFR Activity Assay”), and the mixture was adjusted with sodium-phosphate buffer (pH 7.0) to a final DHFR activity of 0.40 nmol/min/ml. After a 3-min incubation at room temperature in the presence of 0, 0.5, 0.8, 1.0, 1.5, 2.0, 5.0, 10.0, or 100 nM MTX, the
DETECTION AND CLASSIFICATION OF MTX RESISTANCE

Table 1 Theoretical response to the screening procedure of cells possessing defined mechanisms of MTX resistance

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>3-h incubation</th>
<th>6-h efflux</th>
<th>DHFR level</th>
<th>Apparent turnover no.</th>
<th>Relative activity at 10 nM</th>
<th>Intracellular TMQ/MTX ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX</td>
<td>TMQ</td>
<td>FMTX</td>
<td>MTX</td>
<td>TMQ</td>
<td>FMTX</td>
</tr>
<tr>
<td>Sensitive</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>DHFR overexpression</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Altered DHFR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Decreased transport</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Decreased polyglutamylation</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Activity of DHFR in crude extract in the presence of 10 nM MTX (see text).

RESULTS

[3H]-2'-Deoxyuridine Assay. The effects of MTX, TMQ, and FMTX on DNA synthesis of the parent CCRF-CEM cell line and the MTX-resistant sublines R1, R2, R3, and R4 are shown in Table 2. After a 3-h drug incubation, the four resistant cell lines displayed similar patterns of resistance, although the R1 cell line (DHFR amplification) was somewhat less inhibited by TMQ than the other lines. After a 4-h incubation with MTX followed by a 6-h efflux period, some inhibition of DNA synthesis was still present in the sensitive cell line and possibly in the R1 line. The cells exposed to FMTX had recovered completely. In contrast, DNA synthesis in the sensitive cell line, in the cell line with DHFR amplification R1, and in the transport-defective subline R2, was still completely inhibited after 4 h of TMQ exposure and 6 h of efflux, but in the R3 cell line with both DHFR gene amplification and decreased MTX uptake, complete recovery was evident. DNA synthesis in the cell line expressing the altered DHFR, R4, had also recovered.

The detection of high levels of acquired MTX resistance in CCRF-CEM sublines is without problems, but the establishment of reference values is needed to allow differentiation of “sensitive” and low-level resistant cells with this assay. We reasoned that phytohemagglutinin-transformed lymphocytes of healthy volunteers might be suitable cells to aid in this process since these cells (a) are derived from the same tissue as acute lymphoblastic leukemia; (b) may be expected to show “normal” variations in MTX sensitivity between subjects; and (c) will not exhibit acquired drug resistance or resistance that might be related in any way to the malignant state.

PHA-stimulated lymphocytes of five healthy volunteers were subjected to the assay described above. The results (Table 3) showed little variation. All samples showed less than 10% tritium release in the 1 µM MTX incubation, and less than 50% in the 0.1 µM MTX incubation. After MTX exposure and the 6-h incubation in drug-free medium, the remaining DNA synthesis inhibition was more pronounced than in the sensitive CCRF-CEM lymphoblasts, while the recovery 6 h after FMTX exposure was complete. Again, the effect of TMQ was striking, and DNA synthesis inhibition by this drug persisted after 6 h of incubation in drug-free medium.

DHFR Levels and MTX Titrations. The DHFR activity levels, apparent turnover numbers, and DHFR activity in the presence of 10 nM MTX are shown in Table 2. As previously reported (27), the activity was elevated in the cell lines with DHFR gene amplification R1 and R3 lines, but not in the R2 line. The DHFR activity in the R4 line is somewhat elevated, possibly as a result of the presence of several copies of the altered DHFR sequences in the genome of these cells. The MTX affinity of the DHFRs of CCRF-CEM parent cell line and of the sublines R1, R2, and R3 are similar. The R4 line expressed the altered DHFR of the 3T6 mouse fibroblasts, which is much less sensitive to MTX (Fig. 1).

The DHFR activity levels found even in the unamplified CCRF-CEM lines were considerably higher than those found in most fresh patient cells (see below).

Intracellular accumulation of MTX and TMQ. Intracellular levels of MTX were determined after 30 min and after 4 h of incubation, and intracellular levels of TMQ were determined after 30 min in the presence of 1 µM drug (Table 2). The results showed considerably greater accumulation of TMQ than MTX, which is consistent with the greater potency of the former agent. The intracellular drug accumulation was expressed as pmol inhibitor/10⁸ cells. Since large cells may take up more drug than small cells without achieving higher cytoplasmic drug concentrations, we have used the accumulation of TMQ as an internal standard to correct for differences in cell volumes. TMQ presumably has a similar intracellular distribution as MTX, but enters the cell through a different pathway (28). If the MTX uptake is low in comparison to the TMQ uptake a

* S. Rodenhuis et al., submitted for publication.
DETECTION AND CLASSIFICATION OF MTX RESISTANCE

Table 2. Detection and classification of MTX resistance in cultured and in fresh human leukemic cells

<table>
<thead>
<tr>
<th>Patient diagnostic</th>
<th>3-h incubation</th>
<th>6-h efflux</th>
<th>DMFR (nmol/min/10^6 cells)</th>
<th>Relative activity at 10 mM MTX (%)*</th>
<th>PMol inhibitor/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX (1 µM)</td>
<td>TMQ (0.1 µM)</td>
<td>FMTX (5 µM)</td>
<td>MTX (1 µM)</td>
<td>TMQ (0.1 µM)</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>69</td>
<td>4</td>
</tr>
<tr>
<td>Parent</td>
<td>82</td>
<td>100</td>
<td>18</td>
<td>116</td>
<td>61</td>
</tr>
<tr>
<td>R1</td>
<td>90</td>
<td>115</td>
<td>1</td>
<td>73</td>
<td>124</td>
</tr>
<tr>
<td>R2</td>
<td>100</td>
<td>99</td>
<td>5</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>R3</td>
<td>72</td>
<td>102</td>
<td>7</td>
<td>106</td>
<td>78</td>
</tr>
<tr>
<td>R4</td>
<td>15</td>
<td>89</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>1. ALL/</td>
<td>3</td>
<td>69</td>
<td>1</td>
<td>0</td>
<td>101</td>
</tr>
<tr>
<td>2. ALL/</td>
<td>8</td>
<td>16</td>
<td>13</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>3. ALL/</td>
<td>3</td>
<td>97</td>
<td>3</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>4. ALL/</td>
<td>4</td>
<td>49</td>
<td>9</td>
<td>9</td>
<td>No activity</td>
</tr>
<tr>
<td>5. CMLbc</td>
<td>4</td>
<td>89</td>
<td>3</td>
<td>2</td>
<td>34</td>
</tr>
</tbody>
</table>

* Rate of [3H] release of control cells.
\* Activity in crude cellular extracts.
\* Apparent turnover number of DHFR based on the MTX titration experiments (mol/mol/min).
\* Results are expressed as a percentage of enzyme activity with no MTX present.
\* [TMQ]/[MTX] ratio after 30-min incubation in 1 µM drug.
\* ALL, acute lymphoblastic leukemia; CMLbc, chronic myeloid leukemia in blast crisis; ND, not done.

Table 3. [5-3H]2'-Deoxyuridine tests of phytohemagglutinin-stimulated peripheral blood lymphocytes of five healthy volunteers

<table>
<thead>
<tr>
<th>MTX (1 µM)</th>
<th>3-h drug incubation</th>
<th>4-h drug incubation</th>
<th>6-h efflux</th>
<th>DMFR (nmol/min/10^6 cells)</th>
<th>Relative activity at 10 mM MTX (%)*</th>
<th>PMol inhibitor/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ALL/</td>
<td>9</td>
<td>44</td>
<td>5</td>
<td>7</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>2. ALL/</td>
<td>7</td>
<td>27</td>
<td>1</td>
<td>0</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>3. ALL/</td>
<td>0</td>
<td>29</td>
<td>3</td>
<td>2</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>4. ALL/</td>
<td>3</td>
<td>28</td>
<td>2</td>
<td>2</td>
<td>37</td>
<td>5</td>
</tr>
<tr>
<td>5. CMLbc</td>
<td>0</td>
<td>22</td>
<td>1</td>
<td>1</td>
<td>31</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 1. Methotrexate titration curves of dihydrofolate reductase in crude cellular extracts of leukemic cells. The MTX titration curves were obtained by using crude cellular extracts diluted with sodium phosphate buffer (pH 7.0) to a final enzyme activity of approximately 0.40 nmol/min/ml and a radioassay for DHFR (see “Materials and Methods”). The curve of the CCRF-CEM/parent cell line and of patient 6 are similar. The curve of the CCRF-CEM/R4 subline indicated the presence of an enzyme with decreased affinity for MTX.

transport defect may be suspected. The relation between the [TMQ]/[MTX] ratio in fresh leukemic cells after 30 min of drug exposure in vitro and the tritium release in the [5-3H]2'-deoxyuridine test after 3 h of incubation with 0.1 µM MTX is depicted in Fig. 2. As the [TMQ]/[MTX] ratio increases, DNA synthesis inhibition tends to be less inhibited, confirming our earlier results. Presumably as a result of the multifactorial cause of MTX resistance and the relatively small number of patients investigated, this correlation did not achieve statistical significance.

The intracellular concentration of MTX tended to increase during the incubation period in both the cultured cells and in the fresh leukemic cells. CCRF-CEM cells are known to take up MTX rapidly from the medium and equilibrium intracellular levels are achieved within 30 min. The further continuing increase of the intracellular MTX concentration is the result of MTX polyglutamylation (43).

Fresh Leukemic Blast Cells. The results of the assay with fresh human leukemic blasts are listed in Table 2. None of these samples exhibited MTX resistance comparable to that of the resistant CCRF-CEM sublines, not even those of patients considered to be “clinically drug resistant” (see “Discussion”). There was, however, considerable variability in DNA synthesis inhibition after incubation with 0.1 µM MTX. In general, the assay required much higher cell densities to obtain sufficient tritium release for adequate counts (10 times the background) than the experiments with the cultured cells (see “Slope” in Table 2).

The DHFR activity levels were highly variable, but no direct relation with (biochemical) MTX resistance could be observed. Dot-blot analysis of DNA isolated from the fresh cells showed no signs of DHFR amplification (not shown). The detection limit for DHFR amplification (not shown). The detection limit for DHFR amplification (not shown). The detection limit for DHFR amplification (not shown). The detection limit for DHFR amplification (not shown). The detection limit for DHFR amplification (not shown). The detection limit for DHFR amplification (not shown).
the cultured cells. The [TMQ]/[MTX] ratio was smaller than 15 in all "sensitive" cells. The intracellular levels of MTX tended to increase between 30 min and 4 h, possibly as a result of polyglutamylation (43).

DISCUSSION

The objective of this investigation was the development of a simple and rapid assay system that would allow the detection of MTX resistance in fresh cells of patients with leukemia due to DHFR overexpression, decreased MTX transport, altered affinity of DHFR for MTX, or decreased polyglutamylation of MTX. In addition, the assay should be suitable for the monitoring of emerging drug resistance in leukemia patients and be able to classify the type of resistance encountered.

The direct validation of an assay of this kind would require the study of patients who are clinically sensitive or resistant to the agent and to relate the results of the assay system to response and clinical outcome. Since patients with leukemia are almost never treated with MTX as a single agent, however, this approach is clearly not practical. We have therefore attempted to test the performance of the assay on sublines of the human lymphoblastic leukemia cell line CCRF-CEM, of which the sensitivity or resistance to MTX is well characterized. The assay clearly does well under these conditions, but it is evident that fresh patient material differs in many respects from cultured material: S-phase percentages and growth fractions are lower and there is much more heterogeneity within natural leukemias than in the experimental ones. Even more importantly, low levels of resistance of only a minority of the leukemic cells may give rise to complete clinical drug resistance after the selective pressure of treatment has caused that particular clone to overgrow the sensitive cells.

The results of the experiments with the CCRF-CEM cell lines show that the [5-3H]-2'-deoxyuridine assay system alone is of some value in the classification of the MTX resistance. For example, in the case of decreased transport of MTX, there is no cross-resistance to TMQ, which is transported into cells using a transport system different from that for reduced folates (28). In the case of DHFR gene amplification, however, some degree of cross-resistance to a low concentration of TMQ (0.1 μM) is evident as a result of the higher inhibitor-binding capacity of the resistant cells. Taken together with the DHFR activity and the intracellular [TMQ]/[MTX] ratio, the system readily identifies cells with transport impairment, DHFR overexpression, or both. The cell line with the structurally altered DHFR, that has decreased affinity for MTX, is immediately recognized by the abnormal MTX titration (Fig. 1). It must, however, be recognized that naturally occurring leukemias may express much lower amounts of altered enzyme together with unaltered enzyme from the second gene. A cell line with such a pattern of DHFR expression, in which both genes are expressed to a similar degree, was not available. Results of others, using similar methods, suggest that MTX titration in conjunction with a radiolabelling assay is sufficiently sensitive to detect abnormal enzymes in fresh leukemia cells (24).

No CCRF-CEM cell line with resistance to MTX because of defective polyglutamylation is currently available. We have chosen the approach of simulating a cell that does not polyglutamate MTX by exposure to γ-fluoromethotrexate. This compound is very similar to MTX in terms of affinity for DHFR and membrane transport, but cannot be polyglutamylated as a result of the fluorine in the γ position of the glutamate moiety (31). Cells exposed to this compound are therefore expected to display only signs of MTX toxicity that are unrelated to polyglutamylation. Since FMTX and unglutamylated MTX efflux rapidly from cells in drug-free medium, DNA synthesis should recover in FMTX-treated and polyglutamate synthase-defective cells within several hours of removal of the drug. The difference in DNA synthesis inhibition observed following efflux between MTX-treated cells and cells treated with an at least equipotent concentration of FMTX should therefore reflect the extent of polyglutamylation in these cells.

Our results with the cultured cells, but also with the PHA-stimulated lymphocytes and with the patient cells, indicate that this concept is valid. Cells exposed for 3 h to 5 μM FMTX showed a similar degree of 3H release inhibition as the cells treated with 1.0 μM MTX, and all cells had recovered from FMTX after the 6-h efflux period (with one exception, patient 2). In some cases, the 3-h exposure to FMTX appeared to be slightly less effective than the MTX exposure. Since the [5-3H]-2'-deoxyuridine tests were done following a 3-h incubation, when some polyglutamylation may already have occurred, it is likely that this itself is an effect of MTX polyglutamylation and may represent inhibition at secondary sites by MTX polyglutamates (16).

The data for the seven patients listed in Table 2 indicate that the assay can be used to study fresh patient samples and that the results of patients differ from those in cell lines in some respects. The 3H-release rates are much lower, probably as a result of considerably lower S-phase percentages. The DHFR activity levels are lower, but the MTX affinity of the enzymes appears to be similar as shown by the apparent turnover numbers and the MTX titration curves (Fig. 1). The drug accumulation values are similar, as are the [TMQ]/[MTX] ratios after 30 min of exposure.

The data of the patients in Table 2 may be interpreted as follows. Patient 1 was a 39-year-old male in third relapse of Philadelphia chromosome-positive acute lymphoblastic leukemia. He had been extensively treated with multidrug regimens and had received MTX systemically as maintenance chemotherapy and intrathecally. He was considered to be clinically MTX resistant. The very high [TMQ]/[MTX] ratio suggests the presence of impaired transport of MTX. The extremely low 3H release rate indicates low activity of thymidylate synthase, which would lead to a slower depletion of intracellular reduced folate pools in the presence of DHFR inhibition. This may explain the finding, that after 3 h of exposure to 1.0 μM MTX a low level of 3H remains, whereas after 6 h of efflux, 3H release is completely blocked. The alternative explanation for low TS activity, low proliferation activity, is unlikely in view of the rapidly rising WBC at the time of sampling. Polyglutamylation seems to be intact. Patient 2 was a 21-year-old female in first relapse of acute lymphoblastic leukemia after a lengthy complete remission. Her leukemic cells appear to be extremely sensitive to MTX in vitro, perhaps reflecting the intrinsic sensitivity of this disease to MTX. The incomplete recovery from incubation with FMTX after efflux may be the result of the same phenomenon. Patient 3 was a 70-year-old female with acute lymphoblastic leukemia associated with the rare t(4;11) translocation. These leukemias are not typical acute lymphoblastic leukemias and show some characteristics of myeloid leukemia (44, 45). The patients are usually less responsive to chemotherapy. This patient had not been treated with MTX but had failed induction chemotherapy with Prednisone, vincristine, and doxorubicin. The cells of this patient were somewhat less responsive to 0.1 μM MTX and there was little evidence of polyglutamylation as shown by the complete recov-
DETECTION AND CLASSIFICATION OF MTX RESISTANCE

very 6 h after MTX exposure. The contribution of the relatively high DHFR level without gene amplification is unclear. Of interest, however, are recent reports of MTX-resistant cultured human leukemia cells which elevated DHFR in the absence of gene amplification (46, 47). Patient 4 was an 18-year-old male with untreated ANLL. His cells displayed "normal" in vitro sensitivity to MTX. Patient 5 was a 12-year-old male with untreated ANLL. His cells were resistant in vitro against 0.1 μM MTX. The high [TMQ]/[MTX] ratio suggests decreased transport. Patient 6 was a 71-year-old female with untreated ANLL evolved from a refractory anemia with excess of blasts. Her cells showed no signs of MTX resistance in vitro, but had apparently stopped DNA synthesis after the 6-h efflux period. Low MTX polyglutamylation can therefore not be excluded. Patient 7 was a 69-year-old female with chronic myloid leukemia in blast crisis. The only prior chemotherapy had consisted of hydroxyurea. Her relative resistance to 0.1 μM MTX may be the result of somewhat decreased MTX update, as suggested by the high [TMQ]/[MTX] ratio.

It is evident that the interpretations of the data sets of the patients described above are somewhat tentative. Many more patients have to be investigated to establish unambiguous reference values for the test system.

It is of interest that, on a molecular basis, TMQ is a considerably more powerful agent than MTX in human leukemic cells. Our data show that this difference in potency is associated with accumulation to about 10-fold higher intracellular levels. In addition, tritium release was still completely inhibited after a 6-h incubation in drug-free medium, with four notable exceptions: the CCRF-CEM R3 cell line (characterized by amplification of the DHFR gene combined with a MTX transport enzyme), the CCRF-CEM R4 line (which expresses an enzyme with decreased affinity for MTX and possibly also for TMQ), and the patients 1 and 3. TMQ uptake was not lower than in the other cases, suggesting that either TMQ was removed more efficiently during the efflux period or the affinity of DHFR for TMQ was decreased. Further research is required to answer this question. The CCRF-CEM R3 line may serve as a useful model for such studies.

ACKNOWLEDGMENTS

The authors are indebted to Dr. G. P. Beardsley, Department of Pediatric Oncology, Yale University School of Medicine, New Haven, CT, for the procurement of bone marrow samples.

REFERENCES

DETECTION AND CLASSIFICATION OF MTX RESISTANCE

Abelson, H. T., Modest, E. J., and Frei, E., III. Effects of methotrexate
esters and other lipophilic antifolates on methotrexate resistant human
37. Narayanan, R., Jastreboff, M. M. J., Bertino, J. R. Development of an
amphotropic, high titer vector expressing the methotrexate resistant, dihy-
of an altered dihydrofolate reductase encoded by amplified genes in cultured
R. T. Natural resistance to methotrexate in human acute nonlymphocytic
40. Yalowich, J. C., and Kalman, T. I. Rapid determination of thymidylate
41. Bertino, J. R., Sawicki, W. L., Moroson, B. A., Cashmore, A. R., and
Eldager, E. F. 2,4-Diamino-5-methyl-6-(3,4,5-trimethoxyanilino)methyl]quinazoline (TMQ), a potent non-classical folate antagonist inhibitor. I.
42. Bertino, J. R., Perkins, J. P., and Johns, D. G. Purification and properties
of dihydrofolate reductase from Ehrlich ascites carcinoma cells. Biochemistry,
polyglutamates in methotrexate- and sequential methotrexate-5-fluorouracil-
44. Arbuth, D. C., Bloomfield, C. D., Lindquist, L. L., and Nesbit, M. E., Jr.
Translocation 4;11 in acute lymphoblastic leukemia: clinical characteristics
45. Stong, R. C., and Kersey, J. H. In vitro culture of leukemia cells in t(4;11)
46. Dedhar, S., Hartley, D., and Goldie, J. H. Increased dihydrofolate reductase
activity in methotrexate-resistant human promyelocytic leukemia (HL-60)
47. Dedhar, S., and Goldie, J. H. Methotrexate-resistant human promyelocytic
leukemia (HL-60) cells express a dihydrofolate reductase with altered prop-
Development of an Assay System for the Detection and Classification of Methotrexate Resistance in Fresh Human Leukemic Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/12_Part_1/6513

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/46/12_Part_1/6513.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.