Elevated Dihydrofolate Reductase and Impaired Methotrexate Transport as Elements in Methotrexate Resistance in Childhood Acute Lymphoblastic Leukemia

By Larry H. Matherly, Jeffrey W. Taub, Yaddanapudi Ravindranath, Susan A. Proecke, So Chun Wong, Phyllis Gimotty, Steven Buck, Joel E. Wright, and Andre Rosowsky

A retrospective study of clinical resistance to methotrexate (MTX) was performed on 29 archival specimens of frozen lymphoblasts obtained from children with acute lymphoblastic leukemia (ALL), including 19 at initial presentation and 10 at first relapse. Blasts were assayed for dihydrofolate reductase and MTX transport by flow cytometry using the fluorescent methotrexate analog, PT430 (Rosowsky et al, J Biol Chem 257:14162, 1982). In contrast to tissue culture cells, patient blasts were often heterogeneous for dihydrofolate reductase content. Of the 19 specimens at initial diagnosis, 7 exhibited dual blast populations, characterized by threefold to 10-fold differences in relative dihydrofolate reductase; the dihydrofolate reductase-overproducing populations comprised 12% to 68% of the total blasts for these specimens. Remission duration intervals for patients exhibiting dual blast populations were notably shorter than for patients expressing a single blast population with lower dihydrofolate reductase (≤9 months vs ≥15 months, respectively), a difference that was statistically significant (P = .045). There was no apparent correlation between expression of increased dihydrofolate reductase at diagnosis and known patient and disease prognostic features (immunophenotype, age, sex, and white blood count). For the relapsed patients, 4 of 10 exhibited dual lymphoblast populations with elevated dihydrofolate reductase. The majority of the patient lymphoblast specimens were entirely competent for MTX transport and, likewise, expressed immunoreactive reduced folate carriers by indirect immunofluorescence staining with specific antiserum to the transporter. Three patients (2 at relapse and 1 at diagnosis) exhibited heterogeneous expression of impaired MTX transport (14% to 73% of blasts). In only 1 of these patients did the majority of the lymphoblasts (73%) show impaired MTX transport and for this specimen, immunoreactive carrier proteins were virtually undetectable. These results suggest that heterogeneous expression of elevated dihydrofolate reductase and impaired MTX transport are important modes of resistance in childhood ALL patients undergoing chemotherapy with MTX and that these parameters may serve as predictive indices of clinical response to MTX.

METHOTREXATE (MTX) is a key drug in the consolidation and maintenance therapies of acute lymphoblastic leukemia (ALL) in children. Moreover, MTX is administered intrathecally in the prophylaxis and treatment of central nervous system leukemia. Although long-term disease-free survival for children with ALL has continued to increase and now approaches 70%, leukemia relapse is not uncommon. For patients who relapse during treatment, the prognosis is very poor.

The current concepts of MTX resistance are derived mostly from studies of cultured cells characterized by high degrees of homogeneity and elevated levels of resistance. Although critical determinants of drug sensitivity and resistance (dihydrofolate reductase [DHFR], MTX membrane transport, and MTX polyglutamylation) were identified in these models, relatively little is known about which mechanisms are clinically relevant. Elevated DHFR9,10 and impaired MTX transport11-13 have been implicated in clinical resistance; however, these studies involved only a few patients and little attempt was made to establish correlations between the changes in these parameters and clinical response. By contrast, the inherent ability of lymphoblasts from patients with ALL to accumulate MTX and MTX polyglutamates appears to be key to the curative properties of current therapies with MTX.14,15 particularly in “good-risk” patients with B-precursor ALL; furthermore, decreased synthetic capacities for MTX polyglutamates in T-cell ALL blasts may contribute to the poorer prognosis in T-ALL versus B-precursor ALL.16-18 In certain instances, these differences in MTX polyglutamate synthesis may be secondary to changes in drug influx or DHFR binding capacities.16

The recent use of flow cytometry to study P-glycoprotein expression19,20 and daunomycin efflux16 shows the power of this methodology for detecting low levels of resistance in heterogeneous tumors. Likewise, fluorescent analogs of MTX (fluoresceinated MTX [F-MTX] and PT430 [N-(4-amino-4-deoxy-N'6-methylpteryloyl)-N'-(4'-fluoresceinio-carbamyl)-(L-lysine)] bind quantitatively to cellular DHFR and provide a means of detecting tumor populations with differing DHFR levels by flow cytometry.17-20 Competitive displacement of DHFR-bound fluorochrome by added MTX is a direct reflection of the capacity for MTX membrane transport.8,19 On this basis, PT430 was recently used to assay MTX transport in 17 patients with untreated or relapsed
leukemia; although impaired MTX uptake was detected in lymphoblasts from 2 patients with ALL, no evidence for DHFR overproduction was described.

The present report summarizes the results of a retrospective study of MTX resistance in childhood ALL involving changes in DHFR and MTX membrane transport. Our results (1) show the power of flow cytometry as an analytical tool for detecting MTX resistant phenotypes in vivo and (2) establish the feasibility of applying these methods to archival, cryopreserved lymphoblast specimens with documented patient and disease characteristics. Moreover, they (3) establish the significance of blast cell heterogeneity in clinical MTX resistance because the minor drug resistant lymphoblast subpopulations identified in many of our ALL patients would most likely have gone undetected if other, traditional assay methods (enzyme assay, blot hybridization, and reverse transcriptase-polymerase chain reaction [RT-PCR]) had been applied. Finally, (4) our results further establish the potential role of DHFR overproduction and impaired MTX transport as clinically important modes of resistance in ALL patients undergoing chemotherapy with MTX and suggest that these parameters may serve as predictive indices of clinical response to this and related agents.

MATERIALS AND METHODS

Chemicals. [3',5',7-3H]MTX (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). MTX and trimetrexate were obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD). Both radiolabeled and unlabeled MTX were purified by reversed-phase high-performance liquid chromatography as described previously. Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG was purchased from Tago, Inc (Burlingame, CA). Antiserum to a component of the MTX/tetrahydrofolate cofactor carrier (GM-PTX) was prepared in a female New Zealand white rabbit as previously described. PT430 was synthesized as described earlier. Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum and iron-supplemented serum, which were from GIBCO (Grand Island, NY) and Hyclone Laboratories, Inc (Logan, UT), respectively. Assorted chemicals and biochemicals were purchased from Sigma Chemical Co (St Louis, MO).

Cell culture. The parental K562 erythroleukemia line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 containing 10% heat-inactivated supplemented calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere at 37°C in the presence of 5% CO2/95% air. The K25.22, K200A, K200F, and K500E sublines were selected from parental K562 cells by cloning in soft agar in the presence of MTX (25, 200, 200, and 500 mmol/L, respectively), as previously described. The MTX resistant sublines were grown continuously in the presence of MTX; before assay of H-MTX uptake or treatment with PT430 (see below), cells were cultured for 3 to 5 generations without MTX. All cell lines were routinely subcultured every 72 to 96 hours. Cell numbers were determined by direct microscopic counting with a hemacytometer.

For cytotoxicity determinations, cells were cultured in 24-well culture dishes at 50,000 to 70,000 cells/mL in 2 mL of complete RPMI 1640 containing 10% dialyzed fetal bovine serum. Cells were counted after 72 hours. IC50 values were calculated as the concentrations of MTX necessary to inhibit cell growth by 50% compared with control cells grown under identical conditions, except that the growth inhibitor was omitted.

Assay of 3H-MTX uptake and DHFR binding in cultured cells. For membrane transport measurements in cultured cell lines, log-logarithmically growing cells were washed with Dulbecco’s phosphate-buffered saline (DPBS) and suspended into Hanks’ balanced salts. Transport assays were performed at 0.5 μmol/L 3H-MTX from 0 to 210 seconds, exactly as previously described. DHFR enzyme levels were quantitated by measuring the extent of H-MTX binding in the presence of 100 μmol/L NADPH, after chromatography of cell-free extracts on columns of Biogel P6 (200 to 400 mesh) by rapid centrifugation.

Patient specimens. The studies were performed on randomly selected archival cryopreserved leukemic blast cells obtained as bone marrow aspirates from children with ALL at initial diagnosis or first relapse. Patients were treated at the Children’s Hospital of Michigan (CHM) from 1982 to 1993 on Pediatric Oncology Group (POG) treatment protocols (POG 8035, 8025, 8493, 8602, 8617, and 9006) or on a CHM T-cell leukemia protocol. Intermediate-dose MTX (1 g/m2), administered intravenously, was a common component of the consolidation phases of these protocols (starting at 4 weeks) along with intrathecal MTX for central nervous system prophylaxis. In addition, oral and intramuscular doses of MTX were administered during maintenance therapy for B-precursor ALL. Relapsed ALL patients were treated on open POG relapse ALL protocols, based on the individual patient’s eligibility.

Blasts were separated from bone marrow by standard Ficoll hypaque density centrifugation at the time of collection to obtain highly purified mononuclear fractions consisting mostly of leukemic blasts. The purified fractions were cryopreserved in RPMI 1640/10% fetal bovine serum/10% dimethyl sulfoxide at −196°C. All specimens were documented for patient age, sex, presenting leukocyte count, percent blasts in marrow, and immunophenotype, as well as for response to therapy. Eight of the more recently diagnosed patients were analyzed by flow cytometry for DNA indices and S-phase percentages (performed at the POG reference laboratory at St Jude Children’s Research Hospital, Memphis, TN). Upon thawing, blasts were suspended in complete RPMI 1640, containing 10% supplemented serum, and viabilities were evaluated microscopically by trypan-blue exclusion. Only samples with at least 80% viability were used for the flow cytometry assays.

Aliquots (generally 200,000 cells) were cyt centrifuged onto poly-lysine-coated slides for indirect immunofluorescence assay of GP-MTX. An additional aliquot (0.5 to 1 x 106 blasts) was used for the PT430 flow cytometry assay, as described below.

PT430 assay of cellular DHFR and MTX membrane transport. Cultured cells or leukemic blasts (1 x 106) were incubated with PT430 for 12 to 14 hours in folate-free RPMI 1640 (GIBCO) containing 10% dialyzed fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics (see above), in the presence of 10 μmol/L thymidine and 60 μmol/L adenosine. Cells were washed with Ca2+-Mg2+-free DPBS at 0°C to 4°C (3×) and resuspended into PT430-free medium at 0.5 to 1 x 106 cells/75 cm2 in the absence of any additions or the presence of MTX or trimetrexate (generally at 0.5 μmol/L). After additional washes (2×) with Ca2+-Mg2+-free DPBS, cells were suspended in Ca2+-Mg2+-free DPBS containing 0.01% fetal calf serum for flow cytometry. Autofluorescence controls were processed identically, except that the PT430 treatment was omitted.

Flow cytometry analysis was performed on 10,000 blasts with a Becton Dickinson FACStar flow cytometer interfaced with a Consort 30 data acquisition and analysis system. Nonviable cells were excluded from the analysis by gating out propidium-iodide–stained cells. Spectral overlap of the green (FITC) into the red (propidium iodide) fluorescence spectrum was electronically compensated. Excitation was at 488 nm; emission was detected at 525 nm.
**Indirect immunofluorescence assay of GP-MTX.** Cells were cyt centrifuged on poly-L-lysine-coated slides and fixed in 70% methanol at -20°C. The slides were stored at -20°C. The slides were cooled to -50°C in 50% acetone/50% methanol and allowed to warm gradually to 18°C to 20°C. The slides were washed with DPBS and then with DPBS containing 3% bovine serum albumin and were incubated for 1 hour with anti-GP-MTX antibody or nonimmune serum (not shown). The slides were incubated with FITC-conjugated goat antirabbit Ig (30 minutes), washed, and overlaid with 50% glycerol in DPBS for visualization with a Zeiss epifluorescence microscope at 100× magnification. Cells were photographed with Kodak TMAX 400 film at 1600 ASA (Eastman Kodak, Rochester, NY).

**Statistical analysis.** Pearson's correlation was computed for the relationships between PT430 cellular fluorescence and both DHFR and transport impairment. The Mann-Whitney test was used to compare patient data for age, white blood counts, percent S-phase, and DNA index for specimens with dual blast populations to those without. The Fisher's exact test was used to compare the distribution of ALL immunophenotype and patient sex between the two groups. The Kaplan-Meier survival curves for remission duration intervals were computed and the logrank test was used to test for equality of the survival curves. The remission duration interval was defined as the time from initial diagnosis to first relapse. All statistical computations were performed in SAS.

**RESULTS**

**Flow cytometry assay of DHFR contents and MTX membrane transport in cultured cells.** A series of K562 erythroleukemia sublines (parent, K22.25, K200A, K200F, and K500E; Table 1) with a range of MTX sensitivities, DHFR contents, and relative MTX transport capacities were used to validate a flow cytometry assay for these parameters. Our experimental methods were similar to those outlined previously by Trippet et al. and Rosowsky et al. and used PT430, a fluorescent lysine analog of MTX that binds avidly to DHFR and readily penetrates both sensitive and transport-impaired cells by diffusion.

After 12 to 14 hours of exposure to 20 μmol/L PT430, cells were suspended into drug-free medium. A large portion of the intracellular PT430 effluxed during the latter interval; however, a significant "noneffluxable" drug component was retained that remained virtually unchanged from 3 to 5 hours impaired cells by diffusion. Consistent with this notion, the fluorescence ratios of noneffluxable PT430/autofluorescence after 5 hours of efflux closely correlated over a 15-fold range with the actual levels of cellular DHFR in these sublines, as determined by direct titration with 3H-MTX (Fig 2, upper panel).

Displacement of DHFR-bound PT430 during efflux by added MTX compared with trimetrexate (a lipid-soluble DHFR inhibitor that penetrates cells by diffusion) provides a sensitive and quantitative, albeit indirect assay for carrier-mediated MTX transport. Hence, only for cells with intact MTX uptake do low levels of added MTX (0.5 μmol/L) displace the PT430 bound to DHFR (Fig 1); conversely, trimetrexate (0.5 μmol/L) displaces enzyme-bound PT430 in both transport-competent and transport-impaired cells (Fig 1, right panels show data for the MTX-resistant K25.22 subline). Higher concentrations of MTX displace PT430 from both wild-type and transport-impaired cells (data not shown).

**Table 1. Characteristics of K562 Sublines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC50 (nM)</th>
<th>DHFR (pmol/mg)</th>
<th>MTX Transport (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>10</td>
<td>2.15</td>
<td>100</td>
</tr>
<tr>
<td>K25.22</td>
<td>29</td>
<td>8.24</td>
<td>68</td>
</tr>
<tr>
<td>K200A</td>
<td>380</td>
<td>10.10</td>
<td>25</td>
</tr>
<tr>
<td>K200F</td>
<td>430</td>
<td>29.60</td>
<td>56</td>
</tr>
<tr>
<td>K500E</td>
<td>740</td>
<td>14.19</td>
<td>24</td>
</tr>
</tbody>
</table>

IC50s reflect growth inhibition by MTX over 72 hours; DHFR was titrated with radioactive MTX and excess NADPH; transport was assayed as drug influx from 0 to 210 seconds at 0.5 μmol/L MTX.
MTX RESISTANCE IN CHILDHOOD ALL

For the series of K562 subclones with varying levels of MTX transport, the relative capacities of MTX to displace PT430 (expressed as the ratio of PT430 fluorescence in the presence of added MTX to that in the presence of trimetrexate) closely correlated with \(^3\text{H}-\text{MTX}\) uptake over a 12-fold range (Fig 2, lower panel).

These data with K562 cells resemble those obtained with PT430 or F-MTX in earlier studies.\(^{17-20}\) As long as high cell viabilities were preserved (80% to 99%, as assessed by trypan blue staining), excellent results were obtained when these assays were performed on either fresh cultures or cells that had been cryopreserved in liquid nitrogen.

**Flow cytometry assay of DHFR content and MTX transport in patient specimens.** Experiments were performed to assess the possibility of extending these experimental approaches to the study of clinical resistance to MTX in children with ALL. Based on studies showing the feasibility of PT430 binding and displacement assays with cryopreserved cells, we began a retrospective analysis of archival specimens of lymphoblasts in bone marrow aspirates from children with ALL. A total of 47 specimens were retrieved, of which 29 (from 28 patients; Table 2) showed sufficient viabilities (>80%) for flow cytometry assay of DHFR content and MTX transport.

Flow cytometry analyses were performed on 19 specimens obtained at initial diagnosis and 10 obtained at first relapse. For only 1 patient (specimens 10 and 27 in Table 2) were paired samples obtained at both diagnosis and relapse. All children were treated at Children’s Hospital of Michigan from 1982 to 1993 with chemotherapy for which MTX was an important component during the consolidation and maintenance phases of therapy. From this patient population, 18 had B-precursor ALL, 4 mature B-cell ALL, and 6 T-cell ALL; 20 patients were male; the median age was 5 years (range, 3 months to 15 years). Initial white blood counts (WBC) ranged from 1,400 to 684,000/μL; WBCs were greater than 20,000/μL in 9 of 18 patients with B-precursor ALL, 4 of 6 patients with T-ALL, and 3 of 4 patients with B-ALL. The median percentage of blast cells in bone marrow samples was 93% (range, 61% to 100%). Twenty-seven of the patients experienced complete remission during induction therapy; 1 patient (no. 28) experienced induction failure and went into remission with second-line chemotherapy. The detailed characteristics of the patient population used for our studies are summarized in Table 2.

Whereas the cultured lines were highly homogeneous with regard to DHFR content (Fig 1), patient blast populations were often heterogeneous. Figure 3 illustrates typical heterogeneity in which two distinct blast populations are easily resolved, representing 78% and 22%, respectively, of the total and differing in DHFR content by approximately 10-fold. Altogether, 11 patients exhibited dual blast populations with differing DHFR contents (7 B-precursor, 3 T-cell, and 1 B-cell). Of these, 7 were obtained at diagnosis (4 with B-precursor, 2 with T-ALL, and 1 with B-ALL) and 4 at relapse (3 with B-precursor and 1 with T-ALL). The “high” DHFR population comprised 12% to 84% of the total for these lymphoblast specimens; DHFR levels varied by threefold to 10-fold between the blast populations (Figs 4 and 5).

Interestingly, the remission duration intervals (corresponding to the time until first relapse or study completion) for the 19 newly diagnosed patients (all immunophenotypes expressing dual DHFR populations were much shorter than for patients expressing a single blast population with lower DHFR (Fig 6 shows a Kaplan-Meier survival analysis of these data). Six of seven patients from the former group relapsed within 9 months of diagnosis (labeled “dual” in Fig 6). By contrast, 9 of 11 of the “single” DHFR group experienced remission duration intervals of at least 15 months (Fig 6). Five of these latter children are disease-free and are currently off treatment. The difference between these groups was statistically significant (\(P = .045\)).

Although an insufficient number of patients was available to perform these analyses for each ALL immunophenotype, these trends were, nonetheless, preserved when all 18 B-precursor ALL patients were grouped together (including both newly diagnosed and relapsed patients; data not shown). This,
of course, assumes that the dual DHFR populations in relapsed patients were also present at diagnosis.

**Assay of MTX membrane transport by flow cytometry and GP-MTX expression in ALL lymphoblasts.** All of the blasts for 17 of 19 patients at diagnosis and for 9 of 10 patients at relapse appeared competent for MTX transport (Figs 4 and 5, respectively), as reflected in the facile displacement of PT430 bound to DHFR by MTX. This was associated with the detection of immunoreactive MTX/reduced folate carrier, designated GP-MTX, by indirect immunofluorescence staining of cytocentrifuged blast preparations (Table 2 and Fig 7). Some variation in the intensities of immunofluorescence staining was observed among the samples (Table 2).

Of the 3 patients (2 at diagnosis and 1 at relapse; specimens 12, 14, and 24) whose blasts (14% to 73% of the total blasts) exhibited significantly impaired MTX transport, all had elevated DHFR. Whereas immunofluorescence staining with GP-MTX–specific antiserum was observed for 2 of these patients (no. 12 and 14; 14% and 22%, respectively, with impaired transport), GP-MTX was virtually undetectable in specimen 24 (73% of blasts with impaired transport; Fig 7, middle panel).

**DISCUSSION**

Whereas childhood ALL is generally responsive to therapy including MTX, the approaches used to treat this disease have in large part been empirically derived. Today, treatment involves grouping ALL patients into risk categories based on a number of presenting features (age, sex, immunophenotype, etc), so that individuals within a particular group receive similar therapies. Using current therapies, 95% of children with ALL attain a complete remission and more than half remain in complete remission for more than 5 years.

The findings described herein provide strong rationale for the use of additional, mechanistically based prognostic indicators in the design of therapy including MTX. Our data argue for a key role for elevated DHFR in inherent MTX resistance in childhood ALL, as reflected in the close associations between the levels of target enzyme in blast subpopulations at presentation and the duration of first remission.

---

**Table 2. Patient and Disease Characteristics**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Type</th>
<th>Stage</th>
<th>Age (yr)*</th>
<th>Sex</th>
<th>WBC†</th>
<th>RD (mo)†</th>
<th>GP-MTX‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T-cell</td>
<td>Diagnosis</td>
<td>3.5</td>
<td>M</td>
<td>72,000</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>2.5</td>
<td>M</td>
<td>684,000</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>B-cell</td>
<td>Diagnosis</td>
<td>15.0</td>
<td>M</td>
<td>89,000</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>4.0</td>
<td>M</td>
<td>36,000</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>3.5</td>
<td>F</td>
<td>16,500</td>
<td>88+</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>B-cell</td>
<td>Diagnosis</td>
<td>8.0</td>
<td>M</td>
<td>56,000</td>
<td>73+</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>0.7</td>
<td>F</td>
<td>17,900</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>T-cell</td>
<td>Diagnosis</td>
<td>14.0</td>
<td>M</td>
<td>37,000</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>4.0</td>
<td>F</td>
<td>5,400</td>
<td>68+</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>4.0</td>
<td>F</td>
<td>23,000</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>4.5</td>
<td>M</td>
<td>1,500</td>
<td>65+</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>3.5</td>
<td>F</td>
<td>9,800</td>
<td>63+</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>7.5</td>
<td>M</td>
<td>31,500</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>3.5</td>
<td>F</td>
<td>38,000</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>8.0</td>
<td>M</td>
<td>4,000</td>
<td>59+</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>4.0</td>
<td>M</td>
<td>26,500</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>B-precursor</td>
<td>Diagnosis</td>
<td>6.5</td>
<td>F</td>
<td>107,000</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>18</td>
<td>B-cell</td>
<td>Diagnosis</td>
<td>10.0</td>
<td>M</td>
<td>1,800</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>19</td>
<td>T-cell</td>
<td>Diagnosis</td>
<td>6.0</td>
<td>M</td>
<td>515,000</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>15.0</td>
<td>M</td>
<td>43,600</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>2.0</td>
<td>F</td>
<td>304,000</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>5.0</td>
<td>M</td>
<td>4,000</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>2.5</td>
<td>M</td>
<td>5,900</td>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>T-cell</td>
<td>Relapse</td>
<td>6.0</td>
<td>M</td>
<td>79,000</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>T-cell</td>
<td>Relapse</td>
<td>4.8</td>
<td>M</td>
<td>3,800</td>
<td>6.5</td>
<td>ND</td>
</tr>
<tr>
<td>26</td>
<td>T-cell</td>
<td>Relapse</td>
<td>10.0</td>
<td>M</td>
<td>1,400</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>27</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>4.0</td>
<td>F</td>
<td>23,000</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>28</td>
<td>B-cell</td>
<td>Ind. failure</td>
<td>15.0</td>
<td>M</td>
<td>596,000</td>
<td>—§</td>
<td>2</td>
</tr>
<tr>
<td>29</td>
<td>B-precursor</td>
<td>Relapse</td>
<td>10.0</td>
<td>M</td>
<td>3,800</td>
<td>19.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* At diagnosis.
† Units for WBCs are cells per microliter.
‡ Remission duration intervals (RD) correspond to the time from diagnosis to first relapse (expressed in months) or study completion (expressed as months +). In our analysis, specimens 5, 6, 9, 11, 12, and 15 are from patients who are currently disease-free and off treatment.
§ GP-MTX fluorescence was scored from 0 (no fluorescence) to 3 (most fluorescence).
|| Specimens 10 and 27 were from the same patient.
§ Patient experienced induction failure; specimen was obtained approximately 1 month after diagnosis.
A role for impaired MTX membrane transport in clinical resistance to MTX was also suggested; however, because of its lower frequency, it was impossible to establish any direct relationship to remission intervals for the small number of patients studied. Furthermore, no obvious correlations could be demonstrated between either of these resistance markers and various patient and disease prognostic features (immunophenotype, age, sex, and WBC). Although data for the DNA indices and percentage S-phase cells were available for only 8 patients at diagnosis (not presented), for these specimens there were no apparent correlations between these parameters and DHFR levels. Of course, differences in S-phase distribution between blast populations could still contribute to variations in the levels of DHFR in our flow cytometry assay for other specimens.

It is interesting that, for T-cell ALL (with a less favorable prognosis, compared with B-precursor ALL), 3 of 6 children (3 studied at diagnosis and 3 at relapse) exhibited marked increases in DHFR levels; 1 of these patients also showed impaired MTX transport. Because in only 1 of 28 patients were paired samples at both diagnosis and relapse available, the relationship of these resistance markers to acquired MTX resistance could not be evaluated. Although for this patient, neither transport nor DHFR significantly changed upon relapse, both DHFR and transport-based resistance markers were detected in lymphoblasts obtained at relapse in other patients.

Our ability to detect resistance markers in patient lymphoblasts was possible in part because these functional assays of DHFR binding and MTX transport were sufficiently reliable and sensitive to measure small changes in these parameters. Moreover, flow cytometry analysis resolves heterogeneous mixtures of lymphoblasts so that even minor subpopulations with increased levels of DHFR and/or diminished capacities for MTX membrane transport can be detected. Previous investigations using Southern hybridizations, likewise, implicated small increases in DHFR as causal elements in MTX resistance in patients, including those with ALL.\(^5\)\(^7\) However, in another study, no evidence of DHFR overexpression was obtained in newly diagnosed or relapsed ALL by either RNAse protection assay or Southern hybridizations.\(^3\)\(^2\) Both quantitative and qualitative differences between the conclusions from these earlier reports and those from the present study likely reflect the abilities of the methodologies used to resolve heterogeneous tumors and detect minor drug resistant subpopulations. In this study, the
low level DHFR overproduction in the mixed population specimens would have likely gone undetected if such "population-averaging" approaches were used for analysis. It is notable that Trippett et al9 used a nearly identical flow cytometry assay to that described herein and detected impaired drug uptake in two patients with ALL after chemotherapy with MTX. However, the investigators did not comment on the type of heterogeneity observed in our study nor was there any mention of elevated DHFR levels in any of the 17 leukemic blast specimens examined.

Other mechanisms of MTX resistance have been described in vitro including kinetically altered DHFR13-35 and diminished MTX polyglutamate synthesis.36-38 Neither of these mechanisms are directly evaluable by the flow cytometry assay described here. In a particular tumor, resistance to MTX is often multifaceted and the complicated interrelationships amongst drug uptake, binding to DHFR, and MTX polyglutamate synthesis are not obvious. This is exemplified by reports of MTX resistant tumor cells incapable of synthesizing MTX polyglutamates in vitro, often with a defect in MTX transport.39-41 However, in other cases, decreased MTX polyglutamylation in drug-resistant tumors occurs independently of MTX transport.35-38 In a similar fashion, the striking relationship between MTX polyglutamate synthesis and disease prognosis in B-precursor ALL,5,39 or differences in polyglutamate synthesis between B-precursor and T-lineage ALL,11,13 may reflect variations in drug influx (hence, MTX substrate available for polyglutamate synthesis) or DHFR levels (DHFR-bound MTX is not a substrate for folylpolyglutamate synthetase, in addition to changes in folylpolyglutamate synthetase catalytic activity.11

Finally, data were presented that further shed light on the mechanistic bases for transport-based resistance to MTX. Previous reports described decreased levels of the MTX/tetrahydrofolate carrier in transport-impaired K562 cells by Western blotting42 or indirect immunofluorescence staining.42 In the present study, 26 of 29 patient lymphoblasts samples were competent for MTX transport. When 22 of these specimens were assayed for the transporter by indirect immunofluorescence staining, all expressed detectable GP-MTX. Whereas 3 specimens exhibited impaired transport, only for the 1 in which the majority (73%) of the blasts were transport-impaired could a significant downregulation of carrier levels be detected.

In summary, our results demonstrate the facility by which flow cytometry can be used to detect MTX resistance markers in lymphoblasts from children with ALL. Although particularly amenable to the detection of MTX resistant leukemias, these methods should be adaptable to other malignant diseases as well. The salient findings of our study are that increased DHFR and impaired MTX transport appear to be clinically relevant modes of inherent MTX resistance in ALL and that the heterogeneous expression of these resistance markers is likely to confound the interpretation of most traditional assays for these drug-resistant phenotypes. Our results imply that clinical resistance caused by impaired MTX transport may occur less frequently than that caused by increased DHFR. Although all but the highest levels of impaired transport should be circumvented by elevated plasma MTX (≥10 μmol/L) during consolidation phase, the impact of this resistance mode would be greater in the presence of lower MTX concentrations during the maintenance phase. Whereas the data from 19 patients at diagnosis established a statistically significant association between elevated DHFR levels and the duration of first remission on chemotherapy that included MTX, it is important to note that the patients studied were treated with a variety of protocols dating back 8 to 10 years and no attempt was made to segregate patients in our analysis by immunophenotype or any other prognostic variable. Clearly, it will be important to confirm these findings in a larger patient population to further minimize any potential bias imposed on our results by our limited patient sample and establish their relative significance by multivariate analysis. A planned prospective study will address the possibility
Fig 6. Kaplan-Meier curves of remission duration intervals for ALL patients at diagnosis with single and dual lymphoblast populations with differing DHFR contents. Data are shown for 19 patients whose blasts show dual (n = 7) and single (n = 12) lymphoblast populations at initial presentation, when assayed for DHFR contents by the PT430 flow cytometry assay described in the text. The difference in remission duration curves for these patients was statistically significant (P = .045 by the logrank test).

CCRF-CEM

PATIENT # 24

PATIENT # 10

Fig 7. Indirect immunofluorescence staining of CCRF-CEM cells and lymphoblasts from ALL patients with normal (patient no. 10) and impaired (patient no. 24) MTX transport. Cells were cytocentrifuged onto poly-L-lysine-coated slides, fixed in 70% methanol at −20°C, washed with DPBS/3% BSA, and then incubated for 1 hour with anti–GP-MTX antiserum or nonimmune serum (not shown). The slides were incubated with FITC-conjugated goat anti-rabbit Ig (30 minutes), washed, and overlaid with 50% glycerol in DPBS for visualization with a Zeiss epifluorescence microscope at 1,000× magnification.
of whether these differences in survival in relation to DHFR levels remain significant within the context of current treatment protocols.

ACKNOWLEDGMENT

We thank Darcy Lichlyter for performing the flow cytometry analyses, Julie George for assistance with the statistical analysis, and Daryl Talifero for assistance in preparing this manuscript.

REFERENCES

31. Rothenberg ML, Mickley LA, Cole DE, Balis FM, Tsuruo

From www.bloodjournal.org by guest on August 30, 2017. For personal use only.
MTX RESISTANCE IN CHILDHOOD ALL


42. Anand PV, Ratner SS, Angeles SM, Wong SC, Matherly LH: Correlations between methotrexate transport in assorted (K562) erythroleukemia lines and membrane carrier contents, as assessed by indirect immunofluorescence staining and flow cytometry. Proc Am Assoc Cancer Res 34:267, 1993 (abstr)
Elevated dihydrofolate reductase and impaired methotrexate transport as elements in methotrexate resistance in childhood acute lymphoblastic leukemia

LH Matherly, JW Taub, Y Ravindranath, SA Proefke, SC Wong, P Gimotty, S Buck, JE Wright and A Rosowsky