Defective Transport as a Mechanism of Acquired Resistance to Methotrexate in Patients With Acute Lymphocytic Leukemia

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Although the mechanisms of resistance to methotrexate (MTX) are known in experimental tumors made resistant to this drug, little information is available regarding acquired resistance to MTX in patients. A competitive displacement assay using the fluorescent lysine analogue of MTX, N-(4-amino-4-deoxy-N\(^\text{\text{16}}\)-methylpteroyl)-N\(^\text{\text{+}}\)-(4'-fluorescein-thiocarbamyl)-L-lysine (PT430), was developed as a sensitive method of detection of transport resistance to MTX in cell lines, as well as in blast cells from patients with leukemia. Rapid uptake of PT430 at high concentrations (20 μmol/L) in leukemic blasts resulted in achievement of steady-state levels within 2 hours. Subsequent incubation with the folate antagonists, MTX and trimetrexate (TMTX), which differ in the mode of carrier transport, produced characteristic patterns of PT430 displacement. Flow cytometric analysis of the mean fluorescence intensity in the human CCRF-CEM T-cell lymphoblastic leukemia cell line and its MTX-resistant subline clearly identified the presence of transport deficiency in the resistant subline. Analysis of blasts from 17 patients with leukemia, nine with no prior chemotherapy and eight previously treated with chemotherapy, found evidence of MTX transport resistance in two of the four patients who were treated with MTX and considered to be clinically resistant to the drug. The finding that blast cells of some patients with leukemia considered clinically resistant to MTX is due to decreased MTX transport has important implications for clinical use of this drug and for new drug development.

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TRANSPORT RESISTANCE is a common mechanism of intrinsic or acquired resistance to methotrexate (MTX) in experimental tumors both in vitro and in vivo.\(^1\)\(^-\)\(^6\) Previous studies from this laboratory have shown that low levels of MTX polyglutamate formation, rather than decreased influx, are connected with intrinsic resistance of acute myelogenous leukemia (AML) blasts to MTX as compared with acute lymphocytic leukemia (ALL) blasts.\(^7\) However, little information has been obtained in patients with leukemia regarding acquired resistance to MTX due to this or any other mechanism.\(^8\) In particular, there are inconclusive reports regarding the frequency of this occurrence in the clinical setting, especially in patients with leukemia, for whom MTX is a mainstay of the treatment regimen.\(^9\) Limiting factors that have hindered assessment of MTX transport resistance in patients with leukemia by standard transport assays include the requirement of large numbers of cells for analysis and the lack of internal controls to account for heterogeneity of the blast cell population. Heterogeneity may be expressed as variation in the percentage of blasts per sample and in the proliferative rates within the population that may be manifest as an alteration in cellular uptake of MTX. An additional limiting factor to resistance assessment is the requirement of prerelapse and postrelapse samples for comparative analysis, which may require an extensive follow-up period.

The use of fluorescein MTX (F-MTX) in flow cytometric analysis and its displacement by MTX and trimetrexate (TMTX) can identify defects in the transport of MTX,\(^10\)\(^-\)\(^12\) but slow uptake and low signal to noise ratios have led us to abandon this approach in the study of fresh blasts from leukemia patients (unpublished observations). On the basis of previous work indicating that the use of an alternative F-MTX analogue, N\(^\text{\text{+}}\)-(4-amino-4-deoxy-N\(^\text{\text{16}}\)-methylpyrroli)-N\(^\text{\text{+}}\)-(4'-fluorescein-thiocarbamyl)-L-lysine (PT430), may have advantages over F-MTX for the detection of defects in the transport of MTX in cultured cells,\(^13\)\(^-\)\(^15\) we evaluated this assay for use in fresh blasts from patients with leukemia. This report describes the flow cytometric assay and summarizes our results in established human leukemia cell lines (CCRF-CEM parent and an MTX transport-resistant subline) and in leukemic cells from patients. Two of four patients in relapse after treatment with chemotherapy regimens that included MTX were found to be markedly impaired in their ability to take up MTX.

MATERIALS AND METHODS

Chemicals. PT430 was synthesized as previously reported.\(^12\) MTX was obtained from Lederle Laboratories, Carolina, Puerto Rico. TMTX glucuronide was obtained from Warner-Lambert/Parke-Davis, Ann Arbor, MI.

Media and sera for tissue culture were purchased from Mediatech, Calabasas, CA, and Gibco, Grand Island, NY.

Cell lines. CCRF-CEM human leukemia cells and the MTX transport-resistant subline, CEM/T, were propagated in RPMI 1640 medium supplemented with 10% horse serum and 0.2 mmol/L (29.2 mg/mL) L-glutamine at 37°C in a 5% CO\(_2\) atmosphere.\(^16\)

Preparation of patient samples. Blasts were separated from peripheral blood and bone marrow by Ficoll hypaque density centrifugation. Cells were washed twice with RPMI 1640 medium, resuspended in growth medium supplemented with 30 μmol/L hypoxanthine to a density of 2 × 10\(^5\) cells/mL, and incubated for 12 to 24 hours at 37°C in a 5% CO\(_2\) incubator. Viability was...
determined by trypan blue exclusion. All samples analyzed had greater than 80% viability.

Flow cytometry. A Coulter Electronics Epics 752 flow cytometer (Coulter, Hialeah, FL) was used to determine fluorescence intensity. Excitation at 488 nm from a 500 mW argon laser was used. The emission filter was a 525 bandpass filter. Before each analysis, alignment was standardized with 10-μm fluorospheres (Coulter) on channel 100 at a linear gain 5. Mean fluorescence intensity per cell for 20,000 cells was determined with the aid of the computer program MDADS.

PT430 competitive displacement assay. MTX uptake was determined by measurement of the change in fluorescence intensity due to competitive displacement of PT430. A total of 3 x 10⁶ cells was suspended in growth medium supplemented with 30 μmol/L hypoxanthine and aliquoted into six fractions of 5 mL each in 25-cm² tissue culture flasks. The assay is run in duplicate when sufficient blasts are available (> 6 x 10⁶ cells). Viability of the cells was determined before analysis by trypan blue exclusion with a cut off established at 80% or greater for analysis. A 2-mmol/L stock solution of PT430 in 10 mmol/L Na₂HCO₃ buffer was prepared, and 50 μL was added to aliquots 2 through 4. Purity of the PT430 was 99% as confirmed by high-performance liquid chromatography (HPLC) analysis. Following addition of PT430, cells were shielded from direct light exposure. PT430 was not added to aliquot 1, which served as the control for autofluorescence. Aliquots 1 to 4 were incubated for 2 hours at 37°C under 5% CO₂. The remaining cells were used for surface marker determination by direct immunofluorescence staining.

Following incubation, cells from each aliquot were transferred to 15-mL conical tubes and washed twice with phosphate-buffered saline (PBS) prewarmed to 37°C. The cells were resuspended in 5 mL of fresh growth medium and incubated for 30 minutes at 37°C under 5% CO₂ to allow for efflux of the exchangeable component of PT430. Cells were pelleted and the supernatant decanted. Each aliquot was washed twice with PBS, then resuspended in 5 mL of competitive displacement buffer containing 10% dialyzed fetal bovine serum, 20 mmol/L HEPES, and 225 mmol/L sucrose, pH 7.4. Cells were transferred to 25-cm² tissue culture flasks to which the competing ligands MTX and TMTX were added as follows: aliquot 3, 50 μL of 5 x 10⁻³ mol/L MTX; aliquot 4, 50 μL of 3 x 10⁻³ mol/L TMTX. Competitors were not added to aliquots 1 and 2. Aliquot 2 is the control for peak uptake of PT430. All samples were incubated for 2 hours at 37°C under 5% CO₂. Following incubation with MTX or TMTX, cells were washed twice with ice-cold PBS and pelleted in a refrigerated RC-3B centrifuge. Cells were kept at 4°C throughout washing to prevent leakage of PT430. Cells were resuspended in ice-cold PBS at a density of 1 x 10⁸ cells/mL then analyzed by flow cytometry. Mean fluorescence intensity was measured on a population of 20,000 cells and percent change in mean fluorescence (%) displacement was calculated.

Direct immunofluorescence staining. To identify and quantify displacement in the blast cell population, detection of surface antigens was determined by direct immunofluorescence staining tenth of 5 x 10⁶ cells from aliquot 5 with Coulter clone fluorescein-conjugated monoclonal antibodies specific for the type of leukemia under analysis, ie, B4 for ALL and MY9 for AML. Aliquot 6 was incubated with the respective isotopic controls. Cells were incubated at 4°C for 30 minutes then washed twice with cold 1% bovine serum albumin. The forward scattered laser light gate was established using aliquots 5 and 6 to determine the blast cell population of interest. After these boundaries were determined, the gate was used to collect events from other aliquots.

RESULTS

Cell line. Parameters for the PT430 competitive displacement assay were established using the CEM human lymphoblastic leukemia cell line and an MTX transport-resistant subline (CEM/T). The length of time of uptake for establishment of steady-state concentrations of PT430 and the concentration of competitor necessary to achieve maximal displacement of PT430 were determined. At high PT430 concentrations (20 μmol/L), steady-state was reached in 2 hours (Fig 1). Initial uptake was rapid and then reached a plateau at 1 to 2 hours in both the CEM cells and the resistant CEM/T subline, although the plateau achieved was slightly lower in the CEM/T line. Incubation with the competing antifolate ligands MTX and TMTX following initial treatment with PT430 and suspension of the cells in drug-free media demonstrated patterns of PT430 displacement that clearly delineated transport defective cells from sensitive cells. Figures 2 and 3 show the results of competition with increasing concentrations of MTX and TMTX, respectively. Minimal PT430 displacement was noted in the CEM/T cell line on incubation with increasing concentration of MTX, whereas the parental CEM cells exhibit marked displacement. The concentration of MTX at which maximal displacement was attained was greater than 0.3 μmol/L. With TMTX, marked displacement of PT430 was seen in both the CEM and CEM/T cell lines. This was consistent with the fact that TMTX does not use the reduced folate carrier. The concentration of TMTX for maximal displacement was also greater than 0.3 μmol/L. More displacement (up to 90%) was noted with longer times of exposure to MTX or TMTX. However, this longer interval increased the time of the assay for clinical samples (up to 12 hours) and did not change the interpretation of the data.

Patient samples. Blasts from 17 patients with untreated or relapsed leukemia were analyzed by this competitive displacement assay (Table 1, Figs 4 and 5). Nine patients (six AML, two ALL, one biphenotypic) were newly diagnosed. Eight (three ALL, one chronic myelogenous leukemia [CML] in ALL blast crisis, four AML) patients were in
Fig 2. Competitive displacement of PT430 by MTX in CEM parental cells (○) and in CEM/T cells (●). Six aliquots of CEM-resistant (CEM/T) and-sensitive lymphoblasts (5 x 10^6/5 mL) were incubated with 50 µL of a 2-mm stock solution of PT430. After a 2-hour incubation at 37°C under 5% CO₂, the cells were washed twice with PBS prewarmed to 37°C and resuspended in 5 mL of fresh growth media, then incubated for 30 minutes at 37°C to allow for efflux of exchangeable (non-dihydrofolate reductase [DHFR]-bound) PT430. They were centrifuged and washed twice with PBS (37°C) and resuspended in 5 mL of competitive displacement buffer containing 10% dialyzed PBS, 20 mm HEPES, and 225 mm sucrose, pH 7.4. Cells were transferred to 25-cm² tissue culture flasks, and either no MTX or MTX in the concentrations indicated was added. The samples were incubated for 2 hours at 37°C under CO₂, transferred to 15-mL conical centrifuge tubes, and washed twice with 10-mL volumes of ice-cold PBS. After the second wash, the pelleted cells were resuspended in 5 mL of ice-cold PBS and analyzed by flow cytometry. This experiment was repeated three times with similar results.

Fig 3. Competitive displacement of PT430 by TMTX in CEM and CEM/T cells. ○, CEM cells; ●, CEM/T cells. See Fig 2 for additional details.

Fig 4. Competitive displacement of PT430 in blast cells from untreated patients with (A) ALL and (B) AML. Black bars represent the percent of intracellular PT430 displaced by MTX; hatched bars represent TMTX displacement. Patient numbers correspond to patients described in Table 1.

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Prior Treatment Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/M</td>
<td>Pre-B-ALL, CALLA+</td>
<td>VCR, P, ASP, 6MP, 6TG, MTX, CTX, Ara-C, DNR, IDA, TTP</td>
</tr>
<tr>
<td>10</td>
<td>14/F</td>
<td>Pre-B-ALL, CALLA+</td>
<td>VCR, P, ASP, CTX, ADR, DNR, Ara-C, MTX, 6MP, 6TG, IDA, Ara-C</td>
</tr>
<tr>
<td>11</td>
<td>4/F</td>
<td>CML, blast crisis, pre-B-ALL, CALLA+</td>
<td>VCR, P, ASP, MTX, VP-16, Ara-C, MTX, VM-26, DNR, Ara-C, Ifos, CP</td>
</tr>
<tr>
<td>12</td>
<td>17/M</td>
<td>T-cell ALL</td>
<td>VCR, P, ASP, CTX, ADR, Ara-C, MTX, VP-16, Ara-C, VM-26, DNR, Ara-C, Ifos, CP</td>
</tr>
</tbody>
</table>

Patients studied at relapse treated with regimens not including MTX

14 53/F AML DNR/Ara-C, M 195
15 61/M AML IDA/Ara-C, Fazarabine, CP/AZA
16 31/M APML IDA/Ara-C, RA
17 68/M AML IDA/Ara-C, M 195

Patients 10 and 13 were treated on relapse with R-NY II; this includes cyclophosphamide, daunorubicin, prednisone, L-asparaginase, intermediate-dose MTX (200 mg/m²) with leucovorin rescue and cytosine arabinoside (see ref 17 for details).

Abbreviations: P, prednisone; VCR, vincristine; asp, L-asparaginase; CTX, cyclophosphamide; IDA, idarubicin; DNR, daunomycin; Ara-C, cytosine arabinoside; Ifos, ifosfamide; VP-16, etoposide; M 195, a monoclonal antibody; RA, all transretinoic acid; CP, cisplatin; AZA, 5 azacytidine; TTP, thiopeta.

relapse. Four (patients 10, 11, 12, and 13 with ALL) had received prior MTX (Table 1). Two of these four patients (patients 11 and 12) showed evidence of marked impairment in the uptake of MTX, comparable to that seen in the CEM/T cell line (Fig 5). Patient 11 had pre-B-ALL with two previous relapses (central nervous system, then bone marrow). Patient 12 had acute blast crisis of CML manifested as pre-B-ALL. Both patients had relapsed during treatment with the retrieval NY II regimen, which uses...
multiple drugs including 200 mg/m² doses of MTX. As noted in Fig 5, displacement in the cells from patient 11 was minimal with MTX (2.9%), but was marked with TMTX (78.6%), suggesting a marked deficiency in the transport of MTX, but not TMTX. Cells from patient 12 showed minimal displacement not only with MTX (5.4%), but also with TMTX (5.4%), suggesting a defect in the transport of both drugs. In studies of [³H]MTX uptake and polyglutamation following a 24-hour incubation, cells from patient 12 showed low uptake (4.3 pmol/10⁷), but good (46%) long-chain polyglutamate formation (glu₃-glut₆), confirming the results of the displacement assay. Patient 14, a patient with AML, previously treated but not exposed to antifolates, was also noted to have a relatively low level of displacement, 11.6% for MTX and 23.2% for TMTX, but not as striking as that of patients 11 and 12.

**DISCUSSION**

As part of a comprehensive program attempting to understand the basis of natural and acquired resistance to MTX in patients with leukemia, it became clear that a rapid, sensitive, and nonradioactive assay system that would detect defects in the transport of MTX in patient samples was needed. Initially, competitive displacement of F-MTX by MTX and TMTX was investigated for this purpose, but in our experience was not useful for clinical samples. In contrast, the use of PT430 resulted in more rapid transport and a higher signal to noise ratio than F-MTX and clearly detected the transport deficiency in the model system used, the resistant CEM line. More importantly, this assay detected a marked abnormality in the transport of MTX in blasts from two of four relapsed ALL patients previously treated with MTX and believed to be clinically resistant to this drug. It is interesting to note that the blasts from one of these patients (patient 12, lymphoid blast crisis of CML) also did not show displacement with TMTX. This drug has been shown to participate in the multidrug-resistant phenotype, and it is possible that this patient was also resistant to anthracyclines and other drugs of this class (vincristine, etoposide) as well as MTX. In addition, blasts from untreated patients with CML in blast crisis frequently demonstrate the MDR phenotype. One patient (patient 14) with AML in relapse who had not received MTX had a TMTX/MTX displacement ratio of 1.9, consistent with a modest impairment of MTX transport (Fig 5).

Several features of the PT430 competitive displacement assay are particularly advantageous. PT430 enters the cell rapidly and binds to dihydrofolate reductase reaching steady-state levels in a short period of time. Exposure of cells to a high concentration of PT430 (20 µmol/L) results in a steady-state level after only 2 hours in leukemic blasts. This affords a distinct advantage over F-MTX, which requires a longer period of exposure (18 hours) to reach steady-state. Since PT430 is unable to undergo polyglutamation, polyglutamation defects can be excluded as a cause for abnormal uptake in analysis by this method. An additional advantage with the use of PT430 is that the linkage of the lysine side chain to fluorescein is via a thioureido group; therefore, it cannot be cleaved to MTX either chemically or enzymatically. Displacement by TMTX and not by MTX in the assay clearly identified MTX transport-impaired blast cell populations. Finally, leukemia-specific markers are used to deal with the problem of percentage of blasts present from sample to sample.

The amount of PT430 displaced in the cell type varies (50% to 60% in untreated ALL, 30% to 58% and 24% to 90% in untreated AML blasts [Fig 4]). Increasing the concentration of the displacing agent (MTX or TMTX) by 100-fold only slightly increased PT430 displacement; however, increasing the time of displacement to 4 hours from 2 hours resulted in 80% to 90% displacement in CEM blasts (data not shown). We chose the 2-hour displacement time for the clinical studies, since the additional 2-hour incubation added to the length of the procedure, lessened blast cell viability, and did not change the MTX:TMTX displacement ratio of PT430.

While additional studies will be required to assess the incidence of reduced uptake as a mechanism of acquired resistance to MTX in patients with leukemia, the finding that blasts from two of four patients, clinically resistant to MTX, had markedly impaired uptake of this drug has important implications for MTX use in the clinic. This finding may also serve to focus efforts in new drug development, eg, on analogues like TMTX, that are taken up by cells by transport mechanisms different from those used by MTX.

**REFERENCES**


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