RAPID COMMUNICATION

Selective Treatment of SCID Mice Bearing Methotrexate-Transport-Resistant Human Acute Lymphoblastic Leukemia Tumors With Trimetrexate and Leucovorin Protection

By João F. Lacerda, Erdem Göker, Albert Kheradpour, Dieter Dennig, Yaroslav Elisseyev, Catherine Jagiello, Richard J. O'Reilly, and Joseph R. Bertino

Impaired transport of methotrexate (MTX) is a common resistance mechanism of tumor cells to this drug. Trimetrexate (TMTX), a second-generation folate antagonist, is still active against MTX-transport-resistant cells because it enters cells by passive diffusion and does not use the reduced folate transport system for cell entry. Therefore, although leucovorin (LV) protects MTX-sensitive cells from TMTX toxicity, MTX-transport defective cells are poorly rescued by LV. Severe combined immunodeficiency mice bearing MTX-transport-resistant CCRF-CEM acute lymphoblastic leukemia tumors were treated with TMTX alone or with the combination of TMTX and LV, with tumor regressions in both groups (P < .001) and without significant toxicity. These results indicate that TMTX with LV protection may be a useful therapeutic regimen for patients with MTX-transport-defective acute lymphoblastic leukemia. Furthermore, resistance to TMTX plus LV may result in reversion to MTX sensitivity.

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MATERIALS AND METHODS

Reagents and drugs. MTX and LV were obtained from Lederle Laboratories (Pearl River, NY) and TMTX from US Bioscience (West Conshohocken, PA). The fluorescent lysine analog of MTX, N-(4-amino-4-deoxy-N10-methylpteroyl)-L-lysine (PT430) was kindly provided by Dr. A. Rosowsky (Dana-Farber Cancer Institute, Boston, MA). The tetrazolium compound (2,3-bis(2-methoxy-4-nitro-5-sulphonyl)-5(phenylamino)carbonyl-2H-tetrazolium hydroxide; XTT) and phenazine methosulfate (PMS) were purchased from Sigma (St Louis, MO).

Cell lines. The human CCRF-CEM MTX-sensitive ALL parental T-cell line (CEM-S), its MTX-transport resistant subline (CEM-T), and cell lines established from mice bearing CEM-S and CEM-T tumors were propagated in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin (complete medium) at 37°C and 5% CO2.

Mice. Pathogen-free C.B-17 scid/scid mice (SCID mice), 6 to 7 weeks old, purchased from Taconic Farm (Germantown, NY), were housed in microisolation cages under specific pathogen-free conditions and received autoclaved food and water.

In vivo studies. SCID mice were inoculated subcutaneously in the right flank with 107 CEM-T leukemic cells and observed twice weekly for development of tumor formation at the site of inoculation, as previously described. The dimensions of the nodules and weight of the animals were evaluated before the institution of treatment and three times per week thereafter. Mice were randomized to receive TMTX alone, at a daily dose of 20 mg/kg for 5 days, or the combination of TMTX and LV, both administered at a dose of 40 mg/kg twice daily for 10 days, once tumors were palpable. A control group did not receive treatment. TMTX and LV were administered intraperitoneally. The mice were killed and autopsied when the tumor mean surface area exceeded 600 mm2 or when the mice were judged to be moribund. Single-cell suspensions from subcutaneous nodules, liver, spleen, thymus, and bone marrow were analyzed by flow cytometry for surface expression of human antigens and by fluorescent in situ hybridization for the detection of human X chromosome. Tumor cells recovered from SCID mice were also studied for sensitivity to MTX and TMTX as well as for their ability to transport MTX by the PT430 competitive displacement assay.

Cytotoxicity assay. In vitro exponentially growing CEM-S and CEM-T leukemic cells and cells recovered from tumor-bearing animals before and after treatment with TMTX or the combination of TMTX plus LV were exposed to various concentrations of MTX or TMTX with and without LV for 72 hours in 96-well plates. To assess cell viability, the reduction of XTT dye (final concentration, 0.2 mg/mL) with PMS was evaluated after 4 hours of incubation with an EL-340 Biokinetcs Reader (Biotek Instruments, Winooski, VT) at a wavelength of 450 nm and 560 nm, as described elsewhere.

PT430 competitive displacement assay. Uptake of MTX by CEM-S and CEM-T leukemic cells, before inoculation and after recovery from SCID mice, was determined by the PT430 competitive displacement assay, as previously described. Briefly, aliquots of 5 × 106 cells were incubated with 2 μmol/L PT430 for 2 hours at 37°C, after which they were washed three times in phosphate-buffered saline (PBS), incubated at 37°C for 30 minutes in complete...
medium to allow for efflux of exchangeable PT430, washed twice in PBS, resuspended in competitive displacement buffer, and incubated with MTX for 2 hours at 37°C and 5% CO2. Thereafter, the cells were washed twice in PBS to remove displaced PT430 and analyzed by flow cytometry. Mean fluorescence intensity was measured and percent displacement of PT430 by MTX uptake was calculated as mean peak fluorescence decrease.

**Flow cytometry.** In vitro cultured cell lines and leukemic cells recovered from SCID mice inoculated with the same cell lines were stained with monoclonal antibodies directed against human CD45, CD2, CD3, CD7, CD4, CD8, CD25 (interleukin-2 receptor α chain [TAC]), CD71, and HLA-DR conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (Becton Dickinson, San Jose, CA). Mouse cells were detected by antimouse CD45 FITC-conjugated monoclonal antibody (Boehringer Mannheim, Indianapolis, IN). The cells were prepared following standard protocols, which have been already described. A FACS scan flow cytometer using LYSIS II software (Becton Dickenson, Sunnyvale, CA) was used for analysis of surface antigen expression and for the PT430 competitive displacement assay.

**Statistical analysis.** The Wilcoxon rank-sum test was used to compare the decrease in tumor mean surface area from day 28 to day 42 in both groups of treated mice (TMTX and TMTX-LV) relative to the group of untreated animals.

**RESULTS**

**Cytotoxicity studies.** In vitro growing CEM-S and CEM-T cells and those recovered from subcutaneous tumors of untreated mice were exposed for 72 hours to various concentrations of MTX, after which growth inhibition was assessed by XTT reduction with PMS (see Materials and Methods). As depicted in Fig 1, the IC50 of MTX was 15-fold higher for CEM-T than for CEM-S cells. CEM-S and CEM-T cells recovered from untreated SCID mice had the same sensitivity to MTX as the original cell lines (Fig 1). The MTX-transport-defective CEM-T cell line was more sensitive to TMTX than the parental, MTX-sensitive CEM-S cell line. At low concentrations of LV, CEM-T cells were not protected from TMTX cytotoxicity and higher concentrations of LV were able to only partly protect this cell line (Fig 2B). In contrast, LV protected CEM-S cells to a much greater degree than CEM-T cells for all the concentrations of LV tested (Fig 2A).

**PT430 competitive displacement assay.** We have previously shown that the PT430 competitive displacement assay can accurately distinguish the capacity of CEM-S and CEM-T cells to transport MTX. No differences in MTX uptake were found between the original cell lines and those recovered from untreated SCID mice. As depicted in Fig 3, there was marked displacement of the MTX-lysine analog, PT430, in CEM-S cells recovered from untreated SCID mice after incubation with MTX (Fig 3A). In contrast, there was virtually no PT430 displacement by MTX in untreated CEM-T cells (Fig 3B).

**Engraftment and treatment of leukemic cells in SCID mice.** All the mice injected with CEM-T cells developed leukemic nodules at the site of inoculation 3 to 4 weeks later. Flow cytometric analysis of single-cell suspensions prepared from tumor nodules showed the same immunophenotype of the
PT430  PT430 + MTX

Fig 3. PT430 displacement by MTX of untreated CEM-S and CEM-T cells. CEM-S cells (A) and CEM-T cells (B) recovered from untreated SCID mice and labeled with PT430 were either untreated (left histogram) or incubated with MTX for 2 hours (right histogram). Thereafter, the cells were washed twice in PBS to remove displaced PT430. Mean fluorescence intensity (abscissa) was evaluated by flow cytometry and percent displacement of PT430 by MTX uptake calculated as mean peak fluorescence decrease (see Materials and Methods).

Resistance mechanisms in CEM-T cells after treatment in vivo with TMTX or TMTX plus LV: reversal of MTX resistance. It was of interest to determine if the tumors that regressed and then regrew after treatment with TMTX or the combination of TMTX and LV had developed resistance to these regimens and to determine the mechanisms of this resistance. Cell cultures were established from subcutaneous tumors from two animals of each treatment group (TMTX and TMTX-LV). After 7 to 10 days of culture, exponentially growing leukemic cells were tested for sensitivity to TMTX, to the combination of TMTX and LV (1 μmol/L), and to MTX. As depicted in Fig 5A, after exposure to TMTX for 72 hours, these cells were now slightly more resistant to TMTX than untreated CEM-T cells. Cells from mice treated either with TMTX or TMTX plus LV had approximately the same sensitivity to TMTX. There was marked reversal of
TMTX cytotoxicity in CEM-T cells recovered from treated animals with the addition of 1 μmol/L LV (Fig 5B).

One possible explanation for these results was that TMTX-resistant cells had been selected and were now capable of transporting LV, being protected from TMTX cytotoxicity. CEM-T cells recovered from animals treated with TMTX and LV were now completely sensitive to MTX (Fig 6); ie, this regimen has selected revertants, presumably now able to transport MTX as well as LV. Tumors from animals treated with TMTX alone were partially sensitive to MTX (Fig 6). The sensitivity profile shows that there may be two populations of cells, with the minor population still showing increased resistance to MTX (Fig 6).

To confirm that TMTX and LV-treated CEM-T cells were now sensitive to MTX as a consequence of selection of transport revertants, the PT430 displacement assay to evaluate MTX uptake was repeated. As depicted in Fig 7, MTX was capable of displacing intracellular PT430 in CEM-T cells recovered from mice treated with TMTX plus LV to the same degree as in CEM-S cells (Fig 3A), showing that these TMTX-LV-treated CEM-T cells were able to now transport MTX.

**DISCUSSION**

The CEM-T cell line is 15- to 20-fold resistant to MTX as a result of impaired MTX uptake.10 As shown in Fig 4,
these cells grow readily in SCID mice; with the inoculation of $10^7$ cells, the tumor take was 100%. We used this model system to test the concept that LV would increase the therapeutic index of TMTX by decreasing host toxicity, without compromising antitumor efficacy. TMTX alone was highly effective in the treatment of this tumor, both in vitro and in CEM-T–inoculated SCID mice. However, in the clinic, two trials with TMTX as treatment for relapsed ALL showed only transient activity, because TMTX dosage was limited due to severe mucositis.11,13

We used a dose of TMTX in mice equivalent to the doses used in humans to treat pneumocystis carinii infection, in combination with LV.13 This pathogen, similar to transport-resistant ALL blasts, is unable to transport LV; thus, LV does not protect this organism from TMTX lethal effects.14 In patients with pneumocystis carinii infection, this dose schedule has been shown to be well tolerated and highly effective.13 In mice, this dose schedule of TMTX (40 mg/kg or 120 mg/m² twice daily for 10 days) is an LD₁₀₀ without LV protection;15 with LV protection there was little or no toxicity observed (weight loss), with complete tumor regression. The dose of TMTX alone used (20 mg/kg/d for 5 days) is the MTD in mice.15 As expected, with single-agent treatment of tumor masses of up to 600 mm³ (consisting of approximately $6 \times 10^6$ cells), 5 of 6 mice in each treatment group had tumor regrowth and cure was limited by the emergence of drug resistance. Two mice from each treatment group received a second course of TMTX and LV with minimal response. Of interest, leukemic cells studied after TMTX treatment had regained sensitivity to MTX (Fig 6), as well as the ability to transport MTX and reduced folates. As expected, these tumor cells were now protected from TMTX cytotoxicity by LV.

The ultimate role of this treatment in patients with MTX-transport–defective malignancies would probably be in combination with other drugs. This would be relatively easy to accomplish because of the lack of toxicity of the TMTX-LV combination. The sequential use of TMTX-LV followed by MTX to eliminate TMTX-LV resistant cells may also be a worthwhile approach. We are currently also exploring the use of this regimen in SCID mice bearing fresh tumor cells from MTX-resistant ALL patients and in other tumors that are defective in retention of MTX, either because of decreased influx or as a consequence of lack of MTX polyglutamylation, a major cause of intrinsic resistance in human malignancies.16

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