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 Güker, Albert Kheradpour, Dieter Dennig, Yaroslav Elissieyev, 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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From the Program of Molecular Pharmacology and Therapeutics and Bone Marrow Transplantation Service, Memorial Sloan-Kettering Cancer Center, New York, NY.

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Address reprint requests to Joseph R. Bertino, MD, Program of Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.

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Inhibition of CEM-S and CEM-T cell growth by increasing concentrations of MTX. In vitro growing leukemic cells (CEM-S [+] and CEM-T [+]) and those recovered from untreated SCID mice (CEM-S [+1] and CEM-T [+1]) were incubated for 72 hours with increasing concentrations of MTX (abscissa). Cell viability and percent growth inhibition (ordinate) were evaluated by the XTT assay (see Materials and Methods).

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 alpha 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 Dickinson, Sunnyvalle, 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 (Fig 2A and B). 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.1 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
TREATMENT OF MTX-RESISTANT LEUKEMIA IN SCID MICE 2677

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 original cells (data not shown). In a preliminary experiment, mice inoculated with CEM-T cells (n = 6) and CEM-S cells (n = 6) were randomized at day 28 not to be treated (n = 3, each cell line) or to receive the combination of TMTX plus LV (n = 3, each cell line). There were tumor regressions only in mice inoculated with CEM-T cells and treated with the combination of TMTX and LV, whereas untreated CEM-T mice and those bearing CEM-S tumors (treated and untreated) did not respond to this treatment and had progressive growth of leukemic tumors (data not shown). In a second experiment shown in Fig 4, 18 mice bearing CEM-T tumors were randomized into three groups of 6 mice. The control group received no treatment (Fig 4A), a second group received TMTX alone (Fig 4B), and the third group received TMTX and LV (Fig 4C). Both groups of treated mice had complete tumor regression (P < .001 for both). The animals that received TMTX alone had complete regression of leukemic tumors after the last day of treatment (day 33), whereas mice that received the combination of TMTX and LV did not have palpable tumors after 8 days of treatment (day 36). Five of six mice in each treatment group had regrowth of tumors by day 56 (Fig 4B and C) and did not respond to a second course of TMTX plus LV. Both treatment regimens produced minimal weight loss (<2 g).

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. 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.\textsuperscript{11,12}

We used a dose of TMTX in mice equivalent to the doses used in humans to treat pneumocystis carinii infection, in combination with LV.\textsuperscript{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.\textsuperscript{14} In patients with pneumocystis carinii infection, this dose schedule has been shown to be well tolerated and highly effective.\textsuperscript{13} In mice, this dose schedule of TMTX (40 mg/kg or 120 mg/m\textsuperscript{2} twice daily for 10 days) is an LD\textsubscript{100} without LV protection;\textsuperscript{15} with LV protection there was little or no toxicity observed (weight loss), with complete tumor regression. Of TMTX alone used (20 mg/kg/d for 5 days) is the MTD in mice.\textsuperscript{15} As expected, with single-agent treatment of tumor masses of up to 600 mm\textsuperscript{3} (consisting of approximately $6 \times 10^5$ 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 polyglutamlylation, a major cause of intrinsic resistance in human malignancies.\textsuperscript{16}

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Selective treatment of SCID mice bearing methotrexate-transport-resistant human acute lymphoblastic leukemia tumors with trimetrexate and leucovorin protection

JF Lacerda, E Goker, A Kheradpour, D Dennig, Y Elisseeyeff, C Jagiello, RJ O'Reilly and JR Bertino