Inability of Leucovorin to Rescue a Naturally Methotrexate-resistant Human Soft Tissue Sarcoma Cell Line from Trimetrexate Cytotoxicity

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ABSTRACT

A human lymphoblastoid line (RPMI-1788), a methotrexate-sensitive human fibrosarcoma cell line (HT-1080), and a naturally resistant mixed mesodermal human sarcoma cell line with impaired methotrexate polyglutamylation (HS-42), recently established in our laboratory, were used to compare the ability of leucovorin to prevent trimetrexate cytotoxicity with inhibition and an in situ thymidylate synthesis activity assay showed that inhibitory effects of trimetrexate (1 to 10 μM), 24-h exposure, were prevented by 10 μM leucovorin in the RPMI-1788 and HT-1080 cell lines but not in the HS-42 cell line. Total intracellular reduced folates increased about 2-fold in the three cell lines after exposure to leucovorin (10 μM) for 4 h, and after a 6-hour efflux remained elevated (1.5- and 1.3-fold of control levels) in RPMI-1788 and HT-1080 cells but decreased to 80% of control levels in HS-42 cells. Although uptake of leucovorin and levels of N2,N4-methylene tetrahydrofolate achieved after leucovorin administration were similar in RPMI-1788 and HS-42 cells, polyglutamate forms of this coenzyme were less in the HS-42 cells as compared to RPMI-1788 cells. Based on these studies, the combination of trimetrexate with leucovorin should be further investigated as a way to increase the therapeutic index in some patients with soft tissue sarcomas.

INTRODUCTION

MTX is used for treatment of various human tumors, but natural or acquired drug resistance limits its effectiveness in many different tumors (1). At least 5 different mechanisms of acquired resistance to MTX have been observed in experimental and human tumors, including impairment of MTX polyglutamylayation, a mechanism reported in recent years (2-7). This defect results in poor retention of MTX due to a decreased level of long chain polyglutamates in tumor cells. We have recently reported that a major cause of natural MTX resistance in human soft tissue sarcomas was associated with this phenotype (8). New antifolates, which are polyglutamated more efficiently than MTX, such as 10-ethyl-10-deazaaminopterin (9), or do not require polyglutamalation for retention, such as TMTX (10), may be more effective in this disease.

TMTX, also a powerful inhibitor of DHFR, enters the cells by a route distinct from the classical reduced folate transport system and does not form polyglutamates (11). In previous studies we found that some human sarcoma cell lines which are resistant to MTX were still sensitive to TMTX (12).

We postulated that MTX-resistant sarcoma cells with decreased MTX polyglutamylation might also be defective in converting LV to polyglutamate folate coenzymes. If so, LV may protect normal cells from the side effects of TMTX but not protect sarcoma cells with this phenotype. In the present study, we used the HS-42, a naturally MTX-resistant human sarcoma cell line previously shown by us to form low levels of polyglutamates (8), to test this hypothesis. RPMI-1788, a nonmalignant lymphoblastoid cell line, and HT-1080, a MTX-sensitive sarcoma cell line, were used together with HS-42 cells to explore the possible selective action of the combination of TMTX with LV.

MATERIALS AND METHODS

Chemicals. MTX and LV were obtained from Lederle Laboratories. TMTX-glucuronate and [14C]TMTX were supplied by Warner-Lambert/Parke-Davis (Ann Arbor, MI). [5-3H]Deoxyuridine, [3',5',7'-(-n-3H)]-6-fluoro-2'-deoxyuridine-5'-monophosphate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; FPGS, folylpolyglutamate synthase; FH2, dihydrofolate; FH4, tetrahydrofolate; CH2FH4, N5,N10-methylenetetrahydrofolate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BME, 2-mercaptoethanol; ED50, drug concentration required to decrease the cell index to 50% of control; IC50, drug concentration required to inhibit enzyme activity to 50% of control; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum.

Received 6/15/92; accepted 10/7/92.

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3 The abbreviations used are: MTX, methotrexate; TMTX, trimetrexate; LV, leucovorin; FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; FPGS, folylpolyglutamate synthase; FH2, dihydrofolate; FH4, tetrahydrofolate; CH2FH4, N5,N10-methylenetetrahydrofolate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BME, 2-mercaptoethanol; ED50, drug concentration required to decrease the cell index to 50% of control; IC50, drug concentration required to inhibit enzyme activity to 50% of control; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum.
at a final concentration of 2 μCi/ml, and its conversion to thymidine was determined by 3H release at 0, 15, 30, and 45 min. At these times, a 100-μl aliquot of cells was placed in 200 μl of 4% (w/w) trichloroacetic acid with 15% charcoal to stop the reaction. The mixture was centrifuged for 5 min, and the supernatant (100 μl) was added to 5 ml of Ecolume (ICN) scintillation fluid and counted in a scintillation counter (Beckman Model 5801). A blank consisting of medium without cells and drugs was used for background subtraction. The results of the scintillation counts were analyzed by calculating the slope of the 3H release using linear regression. Drug inhibition was expressed as a percentage of the slope of untreated control cells in the assay.

Dihydrofolate Reductase Activity Assay. Exponentially growing cells were harvested, washed twice with cold PBS, and resuspended in 50 mM Tris-HCl (pH 7.5) containing 10% glycerol, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Extracts were obtained by freezing-thawing the cells 3 times followed by centrifugation at 30,000 × g for 30 min at 4°C. The supernatant was used for enzyme assay. DHFR activity was determined as described (16). Briefly, the decrease in absorbance at 340 nm which occurs when NADPH and FH2 are consumed, was measured using a spectrophotometer (Beckman Model DU-65). The protein concentration was measured by Bio-Rad assay.

Uptake of TMTX and LV. Exponentially growing cells were harvested, washed twice with cold PBS, and resuspended in 50 mM Tris-HCl (pH 7.5) containing 10% glycerol, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Extracts were obtained by freezing-thawing the cells 3 times followed by centrifugation at 30,000 × g for 30 min at 4°C. The supernatant was used for enzyme assay. DHFR activity was determined as described (16). Briefly, the decrease in absorbance at 340 nm which occurs when NADPH and FH2 are consumed, was measured using a spectrophotometer (Beckman Model DU-65). The protein concentration was measured by Bio-Rad assay.

Analysis of MTX Polyglutamate Formation. Exponentially growing cells were incubated in complete medium containing 10 μM [3H]MTX at 37°C for 24 h and then washed twice with cold 0.9% NaCl solution. The cell pellets were suspended in 500 μl of boiling 50 mM sodium phosphate, pH 5.5, for 5 min, and centrifuged at 20,000 × g for 10 min at 4°C. The supernatant was stored at -20°C until analysis by HPLC. HPLC analysis of cell extracts was performed using a modification of the method of Cashmore et al. (17). Fractions were collected, and the radioactivity in each 1-ml fraction was measured in a scintillation counter. MTX polyglutamate standards were added to certify the radiolabeled peaks.

Determination of Intracellular Folates. The radioenzymatic assay described by Bunni et al. (18) was used to measure reduced folate pools (CH2FH4, FH2, and FH4) in cell-free extracts. These assays are based on the etrapment of CH2FH4 by TS and [3H]FdUMP to form a stable ternary complex (19). Cells exposed to LV either 4 h or 4 h followed by 6 h efflux in fresh folate-free medium were harvested, washed twice with cold PBS, and suspended in extraction buffer containing 50 mM Tris-HCl, 50 mM sodium ascorbate, 1 mM EDTA, and 0.25 mM sucrose (pH 7.4) at 3 × 107 cells/ml. Cells were then boiled for 10 min and centrifuged at 10,000 × g for 10 min at 4°C. CH2FH4 was estimated by combining 80 μl of cell-free extract with 16 milliunits of TS and 125 nm [3H]FdUMP in a total 200-μl volume. After reaction mixtures were incubated at 25°C for 30 min, the reaction was stopped by the addition of 1% SDS and boiled for 5 min. Aliquots (100 μl) were filtered over a Sephadex G-25 minicolumn, and the eluted fractions were counted to determine bound [3H]FdUMP. FH2 was determined by the same method after conversion to CH2FH4 by the addition of 6.5 mM formaldehyde. FH2 was determined by converting this to FH4 with DHFR in the presence of NADPH (20) and subsequent conversion to CH2FH4 as above.

Determination of Polyglutamates of CH2FH4 and FH4. Polyglutamatates of CH2FH4 and FH4 were determined as described by Priest and Doig (21). Cells were exposed to LV for 4 h and washed twice with cold PBS. Cell-free extracts were then obtained as described above. Ternary complexes were formed among [6-3H]FdUMP, TS, and CH2FH4 and were electrophoresed on a 9% polyacrylamide gel (13 x 21 cm). Equivalent amounts of reaction mixtures were applied to each lane. Gels were fixed in 12.5% trichloroacetic acid for 1 h, followed by treatment with Enhance for 1 h and agitation in water for 30 min, dried, and exposed to Kodak film at -70°C. The resultant fluorograph was displayed as a video image by use of computer-assisted image analysis, and the densities of each area were measured.

Polyglutamate Synthetase Activity Assay. Exponentially growing cells were harvested and washed with 0.9% NaCl solution and then resuspended in 0.1 mM Tris buffer, pH 8.85, containing 0.1 mM BME. Extracts were obtained by freezing-thawing 3 times followed by centrifugation at 3000 × g for 30 min at 4°C, and the supernatant was used immediately for the assay. FPGS activity was determined as described by McGuire et al. (22). Each assay contained, in a final volume of 250 μl, 100 mM Tris-HCl (pH 8.85), 10 mM ATP, 20 mM MgCl2, 20 mM KCl, 100 mM BME, 4 μM [3H]glutamate, enzyme extracts, and substrate (FH4, LV, or MTX). The reaction was stopped at the indicated times with the addition of 1 ml of cold 5 mM glutamate (pH 7.5) containing 25 mM BME, and the polyglutamates that were formed were separated from the glutamate by DE-52 minicolumn chromatography. Enzyme activity is expressed as pmol of [3H]glutamate/h/mg of protein incorporated into the polyglutamate fraction.

Methenyl Tetrahydrofolate Synthetase Assay. This enzyme activity was assayed as previously described (23, 24). Briefly, cells (>109) were harvested, sonicated in 2 ml of homogenization buffer (20 mM BES:20 mM 2-mercaptoethanol:10 mM magnesium acetate:1 mM phenylmethylsulfonyl fluoride, pH 7.0) and centrifuged to obtain supernatants. The reaction is followed by the increase of absorbance at 360 nm using a spectrophotometer (Gilford, Response). The complete reaction mixture contained 50 mM MES, 0.2 mM [6R,5S]-LV, 5 mM ATP, 10 mM magnesium acetate, and 10 mM 2-mercaptoethanol, pH 6.0, in a total volume of 1 ml. One unit of activity is defined as 1 μmol of product formed per min at 30°C, and specific activity is expressed as units per mg of protein.

RESULTS

Properties of Three Cell Lines in Relation to MTX Resistance. ED50 values of MTX measured by growth inhibition in RPMI-1788, HT-1080, and HS-42 were 0.27, 0.17, and 11.0 μM, respectively (24-h exposure). DHFR activity measured in extracts from the 3 cell lines was similar (Table 1). However, when formation of MTX polyglutamates was determined by HPLC, there was a notable lack of long chain polyglutamates (glu3-5) in HS-42 cells, only 4.3% of total intracellular MTX plus MTX polyglutamates (Table 1). In contrast, the formation of long chain polyglutamates in RPMI-1788 and HT-1080 cells was 73.8% and 39% of total MTX plus MTX polyglutamates,

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Table 1 Properties of three cell lines in relation to MTX resistance

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μM)</th>
<th>DHFR activity (μmol/h/mg)</th>
<th>DHFR-bound MTX (pmol/ml)</th>
<th>Polyglutamates (pmol/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1788</td>
<td>0.27</td>
<td>0.08 ± 0.04</td>
<td>ND</td>
<td>87.0</td>
</tr>
<tr>
<td>HT-1080</td>
<td>0.17 ± 0.02</td>
<td>0.09 ± 0.03</td>
<td>1.6</td>
<td>274.0</td>
</tr>
<tr>
<td>HS-42</td>
<td>8.6 ± 0.2</td>
<td>0.11 ± 0.01</td>
<td>2.1</td>
<td>121.2</td>
</tr>
</tbody>
</table>

* Cells exposed to MTX for 24 h, washed, and counted 96 h later.

* ND, not done.

* Numbers in parentheses, percentage.

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respectively. Also, intracellular levels of long chain polyglutamates in HS-42 cells were 10- to 20-fold lower than in RPMI-1788 and HT-1080 cells (Table 1).

Effects of LV on Cell Growth Inhibition by TMTX. The effects of LV on the cytotoxicity of TMTX in the 3 cell lines were examined by growth inhibition after a 24-h exposure to the drugs (Table 2). All 3 cell lines were equally sensitive to TMTX. When LV (10 μM) was added, TMTX cytotoxicity in RPMI-1788 and HT-1080 cells was markedly decreased (Table 2). ED₅₀ values increased 21- and 18-fold compared with that of TMTX alone. In contrast, in HS-42 cells the toxicity of TMTX was not significantly decreased by addition of LV.

Thymidylate Synthesis Activity Assay. An in situ [³H]dexametoyuridine release assay was used to evaluate the effect of LV on inhibition of thymidylate synthesis activity by TMTX in the 3 cell lines (Fig. 1). TS activity in all 3 cell lines was markedly inhibited by TMTX at 1 μM or higher concentrations after a 3-h incubation with drug. The addition of 10 μM LV reversed inhibition as noted in HT-1080 cells, but only reduced slightly the inhibition of thymidylate synthesis activity by TMTX in RPMI-1788 as well as HS-42 cells. However, after 4-h efflux, inhibitory effects of TMTX (1 to 10 μM) were reversed by 10 μM LV in RPMI-1788 but not in HS-42 cells. These results suggested that the lack of rescue by LV in HS-42 was associated with poor retention of intracellular LV.

Uptake of TMTX and LV. At an extracellular concentration of 1 μM, the uptakes of [¹⁴C]TMTX and [³H]LV were measured in the 3 cell lines (Fig. 2). Uptake of TMTX in the 3 cell lines was similar, with a total accumulation at 60 min of 233, 280, and 320 pmol/10⁷ cells in RPMI-1788, HT-1080, and HS-42 cells, respectively (Fig. 2A). However, there was a greater uptake of LV in HT-1080 (65.4 pmol/10⁷ cells at 60 min) compared with that in RPMI-1788 and HS-42 cells (17.2 and 28.4 pmol/10⁷ cells) (Fig. 2B).

Determination of Intracellular Reduced Folates. Total reduced folates, the sum of CH₂FH₄, FH₄, and FH₂, were 24.9, 33.7, and 38.3 pmol/10⁷ cells for RPMI-1788, HT-1080, and HS-42 cell lines, respectively, compared to control when cells were treated with 10 μM LV for 4 h. After a 6-h efflux of cells in folate-free medium to allow efflux of folates, the total intracellular reduced folates increased approximately 2-fold in all 3 cell lines. However, after a further 6-h incubation of cells in folate-free medium to allow efflux of folates, the total intracellular folates retained in HS-42 cells were only 84% of the untreated control level. In contrast, levels of total reduced folates in RPMI-1788 and HT-1080 cells were 36.5 and 48.2 pmol/10⁷ cells, which were 147% and 143% of control levels (Table 3).

As also shown in Table 3, the intracellular levels of CH₂FH₄ increased 2-fold in RPMI-1788 and HT-1080 and 1.5-fold in HS-42 cell lines, respectively, compared to control when cells were treated with 10 μM LV for 4 h. After a 6-h efflux of cells in folate-free medium, CH₂FH₄ levels decreased to 64% of control in HS-42 cells but remained elevated 2- and 1.3-fold in RPMI-1788 and HT-1080 cells, respectively.

Measurement of Folypolyglutamates. The influence of LV on the formation of polyglutamates of CH₂FH₄ and FH₄ was next examined in the 3 cell lines (Fig. 3). In untreated cells, the RPMI-1788 cell line contained penta- and hexaglutamates with small amounts of tetraglutamates. Line HT-1080 contained hexa- and heptaglutamates with a small amount of pentaglutamates. Line HS-42 also contained hexa- and heptaglutamates. After exposure to 10 μM LV for 4 h, in RPMI-1788 cells, there was an increase of tetra- and pentaglutamates as well as of triglutamates. In HT-1080 cells, there were an increase in pentaglutamates and a slight decrease of hexaglutamates. In HS-42 cells, pentaglutamates also increased with a loss of hexa- and heptaglutamates. Total CH₂FH₄ and FH₄ polyglutamate formation was RPMI-1788 > HT-1080 > HS-42 cells in both untreated and treated cells.

Folypolyglutamate Synthetase Activity. To examine whether a low level of folypolyglutamates in HS-42 cells was related to FPGS activity, the activity of FPGS was measured in cell extracts (Table 4). The activity of FPGS in 3 cell lines using FH₄ as the substrate was similar. Also, when the activity of FPGS was determined using MTX and LV as the substrate, no significant difference was observed.

Methylen Tetrahydrofolate Synthetase Activity. This enzyme activity was assayed because a deficiency of this enzyme would lead to decreased intracellular levels of CH₂FH₄ following LV administration. No significant difference was found in the level of this enzyme activity in all three cell lines (0.8, 1.1,
LEUCOVORIN AND TRIMETREXATE CYTOTOXICITY

Table 3  Effect of LV on intracellular reduced folate pools

<table>
<thead>
<tr>
<th>LV 10 μM</th>
<th>RPMI-1788</th>
<th>HT-1080</th>
<th>HS-42</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-h incubation</td>
<td>CH₂FH₄</td>
<td>FH₄</td>
<td>FH₂</td>
</tr>
<tr>
<td>-</td>
<td>6.4 ± 1.8</td>
<td>6.6 ± 0.7</td>
<td>11.9 ± 2.2</td>
</tr>
<tr>
<td>+</td>
<td>16.2 ± 2.3</td>
<td>16.1 ± 3.5</td>
<td>15.5 ± 1.6</td>
</tr>
<tr>
<td>6-h efflux</td>
<td>8.1 ± 2.4</td>
<td>7.7 ± 0.8</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>8.1 ± 3.2</td>
<td>9.2 ± 1.9</td>
<td>11.4 ± 0.9</td>
<td>13.0 ± 1.3</td>
</tr>
</tbody>
</table>

a Cells were incubated in the presence and absence of 10 μM LV for 4 h and assayed for the three reduced folate pools as described in “Materials and Methods.”
b Mean ± SE of two or three different experiments.
c After a 4-h incubation with or without LV, cells were allowed to incubate an additional 6 h in LV-free medium and assayed for reduced folates.

DISCUSSION

Growth inhibition of HS-42 cells by TMTX was not prevented by coincubation with LV after a 24-h incubation followed by incubating cells in drug-free medium for 96 h. In contrast, growth inhibition of RPMI-1788 and HT-1080 cells by TMTX was significantly decreased by LV. The results that were obtained with inhibition measured by in situ TS activity were consistent with growth inhibition studies in that inhibition of TS activity in RPMI-1788 and HT-1080 cells by TMTX was much less in the presence of LV, but not in HS-42 cells, when measured after 4-h incubation in drug-free medium. The inability of LV to prevent growth inhibition by TMTX in HS-42 cells thus appeared to be related to poor retention of intracellular LV and reduced folates derived from this compound.

Reduced retention of LV may result from a decreased uptake of LV, low levels of methenyltetrahydrofolate synthetase or impaired reduced folate polyglutamylilation (25). Uptake of LV in HS-42 cells was comparable to that in RPMI-1788 cells, and an increased uptake of LV was noted in HT-1080 cells. These were no differences observed in methenyltetrahydrofolate synthetase activity cell lines. Levels of CH₂FH₄ that formed after a 4-h exposure to LV in the 3 cell lines were similar, confirming that there was no impairment of the ability of HS-42 cells in taking up and converting LV to CH₂FH₄. However, levels of CH₂FH₄ were significantly less in HS-42 cells compared with those in RPMI-1788 and HT-1080 cells when assayed after 6-h efflux. There was less formation of long chain (glu3-7) polyglutamates of CH₂FH₄ and FH₄ in HS-42 cells after LV administration compared to RPMI-1788 and HT-1080 cells; thus, low retention of LV in HS-42 cells was likely due to decreased polyglutamate steady-state levels. Lack of accumulation of MTX polyglutamate was previously noted in this cell line after incubation with [³H]MTX for 24 h (12).

The intracellular levels of folate polyglutamates are believed to be regulated by the enzyme, FPGS, which is responsible for synthesis, and folylpolyglutamate hydrolase, which breaks down the polyglutamates to the monoglutamate forms (26). FPGS enzyme activities in the 3 cell lines were similar; thus, the low levels of MTX and folylpolyglutamates achieved in HS-42 cells after MTX or LV administration may not be due to an impairment of this enzyme activity as has been reported in other MTX-resistant cell lines (27, 28). Other possible causes for low levels of folylpolyglutamates formed in HS-42 cells are being investigated, including decreased synthesis in whole cells due to decreased availability of magnesium ATP or glutamates (22), an increase in catabolism of polyglutamates (9), or the presence of an inhibitor of FPGS activity present in intact cells.
The RPMI-1788 cell line was selected as a model for normal hematopoietic cells to explore the ability of LV to prevent TMTX cytotoxicity (29). LV protects RPMI-1788 cells from TMTX cytotoxicity, and these cells are able to form long chain polyglutamates from both MTX and reduced folate. Recent studies using purified human bone marrow myeloid precursors indicated that those cells formed long chain polyglutamates of MTX (30, 31). In addition, it has been well established that LV can protect bone marrow from TMTX toxicity (32).

These observations demonstrate the inability of LV to prevent growth inhibition caused by TMTX in naturally MTX-resistant sarcoma cells having impaired MTX polyglutamylation. These studies provide a basis for using TMTX-LV in combination in patients with the HS-42 phenotype to improve the therapeutic index of TMTX.

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