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Reversal of Methotrexate Cytotoxicity to Human Bone Marrow Cells and Leukemic K562 Cells by Leucovorin: Methotrexate Polyglutamates Formation as a Possible Important Factor

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Methotrexate (MTX) is metabolized intracellularly to MTX-polyglutamates (MTX-PGs), which markedly inhibit several folate-dependent enzymes. Polyglutamation defect, therefore, is one of the important factors in drug resistance. In this study, reversal of MTX cytotoxicity by *l*-leucovorin (*l*-LV) was investigated using normal human bone marrow granulocyte progenitor cells (G-CFCs), and MTX-sensitive and -resistant leukemic K562 cell lines; the latter showed diminished polyglutamation. Cytotoxicity of 10^{-7} *M* MTX to G-CFCs was completely reversed by an equimolar concentration of *l*-LV, but with higher MTX concentrations, relatively more *l*-LV was required. The reversal of MTX cytotoxicity by *l*-LV was more effective against bone marrow cells than MTX-sensitive K562 cells; this reversal seemed to be correlated to the total intracellular MTX levels as well as MTX-PG formation (low in bone marrow cells and high in K562 cells). When MTX-sensitive and -resistant K562 cells were incubated with MTX under conditions in which the total intracellular MTX levels of both cells were similar, successful reversal of MTX toxicity by *l*-LV was demonstrated in MTX-resistant cells, but not in MTX-sensitive cells, suggesting that an increase of MTX-PG formation in MTX-sensitive cells may explain the failure of *l*-LV to overcome MTX cytotoxicity. In addition to competitive reversal of MTX cytotoxicity by LV, noncompetitive reversal relating to variable formation of MTX-PGs is suggested to be another important factor in the mechanism of the reversal of MTX cytotoxicity by LV.

Key words: Leucovorin — Methotrexate — Polyglutamate

Methotrexate (MTX) plays an important role in the treatment of many cancers including acute lymphoblastic leukemia, non-Hodgkin's lymphoma and osteosarcoma.[1] MTX, like each of the reduced folates, is intracellularly metabolized to MTX-polyglutamates (MTX-PGs) by the enzyme folylpolyglutamyl synthetase with as many as three to five glutamate groups added in series to the molecule.[2] Synthesis of MTX-PGs has been identified in a number of normal[3–6] and neoplastic cells.[7–9] MTX-PGs persist intracellularly and continue to inhibit dihydrofolate reductase (DHFR) for prolonged periods even after the disappearance of extracellular MTX.[10, 11] In addition, MTX-PGs have recently been reported to inhibit markedly a number of other folate-dependent enzymes including thymidylate synthase (TS)[12, 13] and 5-aminoimidazole carboxamide ribotide (AICAR) transformylase[14] in the *de novo* purine synthetic pathway. Through these pharmacologic researches, major modifications in dosage and schedule of MTX administration have been clinically possible in recent years followed by leucovorin (LV) rescue of normal tissues.[1] On the other hand, defect of MTX-PG formation has been recognized to be one of the prevalent clinical resistance mechanisms.

Since LV (5-formyltetrahydrofolate) does not require reduction by DHFR, as does folate, LV can restore reduced folates in the presence of a DHFR-inhibitor like MTX by by-passing the enzymatic block and repleting reduced folates distal to the block. Recently, instead of *dl*-LV, the *l*-isomer of LV (*l*-LV) has been developed and become clinically available. In this study, we investigate the correlation between MTX-PG formation and the effectiveness of *l*-LV in the reversal of MTX cytotoxicity to normal bone marrow (BM) granulocyte progenitor cells (G-CFCs), and MTX-sensitive and -resistant leukemic K562 cell lines (these two cell lines exhibit good and poor formations of MTX-PGs, respectively). Our results show that the reversal of MTX cytotoxicity by *l*-LV is correlated to not only competition of the two agents for a common membrane transport system, but also variable formation of intracellular MTX-PGs in a noncompetitive fashion.

MATERIALS AND METHODS

Cell preparation BM samples were obtained from healthy adult volunteers after informed consent had been given. Mononuclear cells were separated by Ficoll-

Hypaque (Lymphoprep; Nyegaard, Oslo) gradient centrifugation at 400*g* for 30 min as described previously.[15] After removal of adherent cells by incubating a mononuclear cell suspension on plastic culture dishes (Costar, Cambridge, MA) at 37°C for 60 min, nonadherent (NA) cells were obtained and resuspended in Iscove's modification of Dulbecco's medium (IMDM; Sigma Chemical Company, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS; Mitsubishi Chemical Industries Ltd., Tokyo).

The human erythroleukemic K562 cell line was purchased from the ATCC (Rockville, MD) and was incubated in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% FCS, 2 m*M* glutamine (Nissui, Tokyo), 100 units/ml penicillin and 5 mg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. An MTX-resistant K562 subclone was established from the parent cell line by exposure of cells to gradually increasing concentrations of MTX (provided by the Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD) and was finally cloned by a limiting dilution technique as described previously.[16] These cells showed more than 1000-fold increased resistance to MTX in the initial study,[16] but the degree of resistance gradually declined while the cells were maintained in complete medium without MTX for 1 year or more. At the time of this work, these cells showed approximately 50-fold increased resistance to MTX as compared to the parent cells. Extensive studies of the mechanisms of drug resistance have revealed that defect of MTX-PG formation is still one of the major causes of drug resistance.[16]

Cytotoxicity studies Cytotoxicity of MTX against normal human BM progenitor cells was studied by using a G-CFC assay system.[17] First, NA cells (10^5/ml) were incubated with 10% dialyzed FCS and various concentrations of MTX for 24 h. FCS was dialyzed against 3 changes of a 10-fold excess of phosphate-buffered saline (pH 7.4) at 4°C since the inhibitory effect of MTX was diminished in medium supplemented with non-dialyzed FCS.[18, 19] After incubation, cells were spun down without washing. The supernatant was discarded and replaced with non-dialyzed FCS (final concentration of 15%), methylcellulose (0.8%) and various concentrations of LV. Recently, the biologically active isomer, *l*-LV (Lederle, Japan) has been developed and it was used throughout this study. In our preliminary study, *l*-LV was slightly (≈2-fold) more effective than *dl*-LV as judged from the ability of LV to reverse MTX toxicity to human BM and K562 cell colony formation. Recombinant human G-CSF (Chugai Pharmaceutical Co., Tokyo) was used as a source of colony-stimulating factor at a concentration of 10%. The culture mixture (0.25 ml each) was plated in 24-well tissue culture plates (Costar) in triplicate and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. G-CFC colonies (40 cells or more) were counted after 7 days of incubation, using an inverted microscope.

The parent K562 and MTX-resistant K562/MTX cells were also examined for MTX toxicity using clonogenic assays in the same manner as above. Briefly, these cells (10^3/ml) were incubated with MTX at various concentrations for 24 h, and then without washing an aliquot of supernatant was replaced with FCS, 0.8% methylcellulose and increasing concentrations of *l*-LV. After incubation for 3 to 5 days colonies were counted.

Determination of MTX-PGs For analysis of intracellular MTX metabolism, purified BM myeloid progenitor cells were obtained by using an immune rosette technique that employed a cocktail of monoclonal antibodies against mature granulocytes, monocytes, T and B lymphocytes and erythroid precursors (anti-CD13, anti-CD3, anti-CD20 and EP1, respectively) as described previously.[5] The parent K562 and K562/MTX cells were cultured with 10^{-6} *M* or 10^{-5} *M* ^{3}H-MTX for 24 h. After incubation, cells were harvested to determine total intracellular drug and metabolite levels. MTX-PG profiles were analyzed by using a modification[16] of a high-performance liquid chromatography technique which was originally described by Jolivet *et al.*[7]

RESULTS

The effects of *l*-LV on MTX cytotoxicity to BM G-CFCs at various concentrations of the drugs are shown in Fig. 1. Without *l*-LV rescue, G-CFCs were inhibited to 30% of the control in the presence of 10^{-7} *M* MTX. At 10^{-6} *M* MTX or above, G-CFC colony formation was completely blocked. The inhibitory effect of 10^{-7} *M* MTX on G-CFCs was effectively reversed by an equimolar concentration of *l*-LV. However, when G-CFCs were incubated with 10^{-6} *M* MTX for 24 h, the equimolar concentration of *l*-LV (10^{-6} *M*) rescued only about 60% of G-CFCs from MTX cytotoxicity. Approximately 100-fold excess of *l*-LV was required for complete rescue for G-CFCs treated with 10^{-6} *M* MTX. Furthermore, G-CFCs cultured in 10^{-5} *M* MTX were rescued from MTX cytotoxicity to 70% of the control by 10^{-4} *M* *l*-LV and to 80% by 10^{-3} *M* *l*-LV. These data suggested that the reversal of MTX cytotoxicity by *l*-LV was competitive, but with higher MTX concentrations, relatively more *l*-LV was required.

The effectiveness of *l*-LV on the reversal of MTX cytotoxicity to G-CFCs was compared with that in the case of leukemic K562 parent cells. As shown in Fig. 2, the reversal of MTX cytotoxicity by *l*-LV was more effective on G-CFCs than K562 cells. In contrast to BM cells, K562 parent cells cultured in 10^{-6} *M* MTX were not sufficiently reversed from MTX cytotoxicity even by

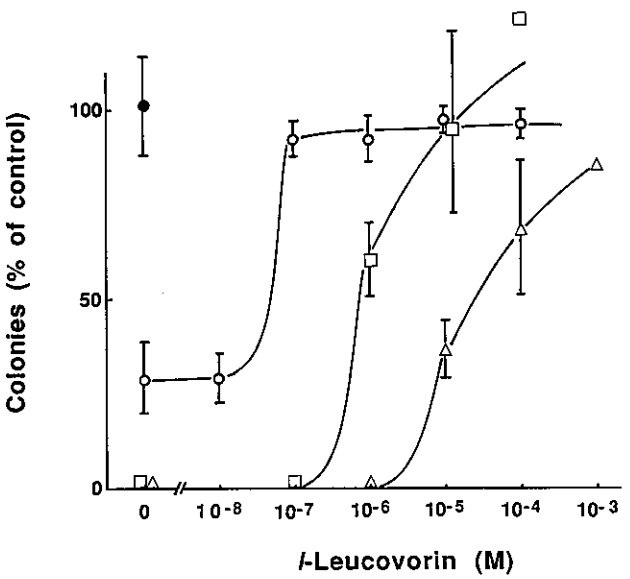

Fig. 1. Reversal of MTX cytotoxicity to normal human bone marrow progenitor cells (G-CFCs) by *l*-leucovorin at various concentrations of the drugs. ●, 10^{-8} *M* MTX; ○, 10^{-7} *M* MTX; □, 10^{-6} *M* MTX; △, 10^{-5} *M* MTX. Each value is expressed as the mean±SD of 3 separate experiments.

a very high concentration (10^{-3} *M*) of *l*-LV. Similar findings, but to a lesser extent, were also seen at 10^{-5} *M* MTX. Finally, the reversing ability of *l*-LV on MTX cytotoxicity to K562 parent cells was compared with that in the case of low-grade (50-fold) MTX-resistant K562/MTX cells; the latter showed MTX-PG formation defect. In these experiments, total intracellular MTX concentrations of both cells were designed to be equal (10^{-6} *M* extracellular MTX levels for K562 parent cells and 10^{-5} *M* for K562/MTX cells). As shown in Fig. 3, successful reversal of MTX cytotoxicity by *l*-LV was demonstrated in K562/MTX cells, but not sufficiently in the K562 parent cells.

To study the causes of differences in the reversal of MTX cytotoxicity by *l*-LV among these cells, the intracellular metabolism of MTX in each cell line was investigated. The different capacities of these cells to convert MTX to MTX-PGs *in vitro* are shown in Figs. 4 and 5. After 24 h incubation with 10^{-6} *M* or 10^{-5} *M* MTX, intracellular accumulation of MTX in BM cells were significantly lower than that in the leukemic K562 parent cells (Fig. 4). In parallel, the formation of MTX-PGs with 3 to 5 glutamate groups was found to be significantly lower in BM cells as compared to K562 parent cells. Figure 5 shows a comparison of intracellular

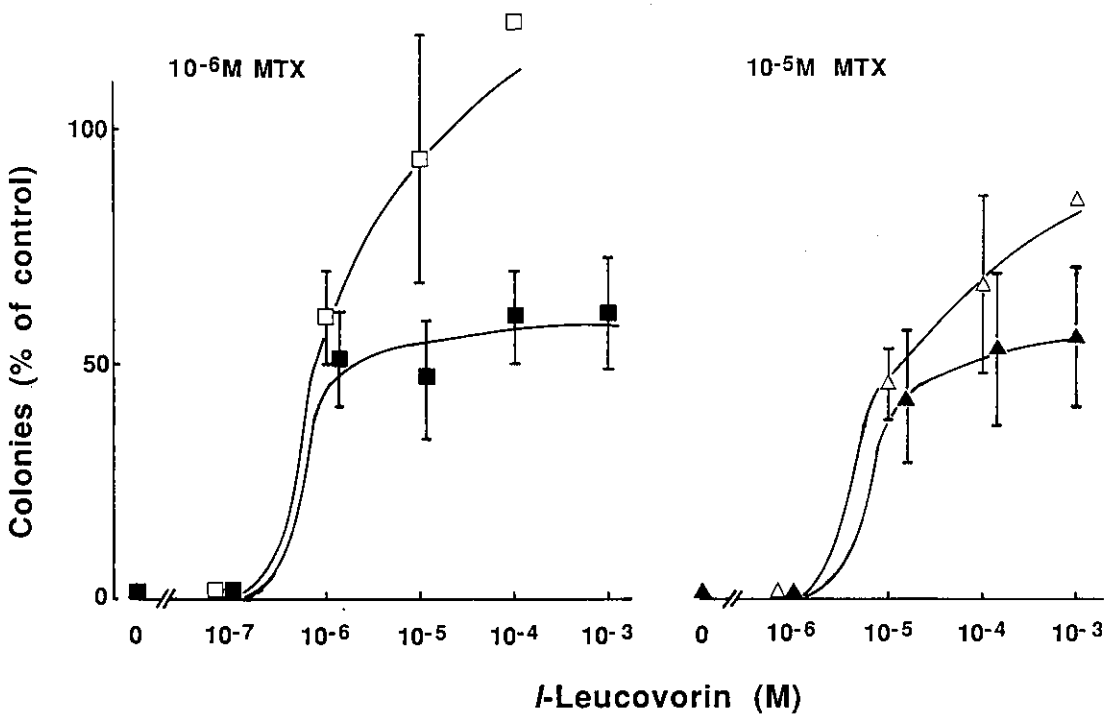

Fig. 2. Reversal of MTX cytotoxicity to bone marrow G-CFCs (□ and △) and K562 cells (■ and ▲) by *l*-leucovorin at various concentrations of the drugs. Each value is expressed as the mean±SD of 3 separate experiments.

MTX-PG profiles between the K562 parent and K562/MTX cells. Under conditions in which the total intracellular MTX levels of both cell lines were designed to be similar, MTX-PG formation was diminished in the K562/MTX cell line; this cell line showed remarkable effectiveness of *l*-LV on the reversal of MTX cytotoxicity. In contrast, K562 parent cells showing an increase of MTX-PG formation were not sufficiently reversed by *l*-LV from MTX cytotoxicity (Fig. 3).

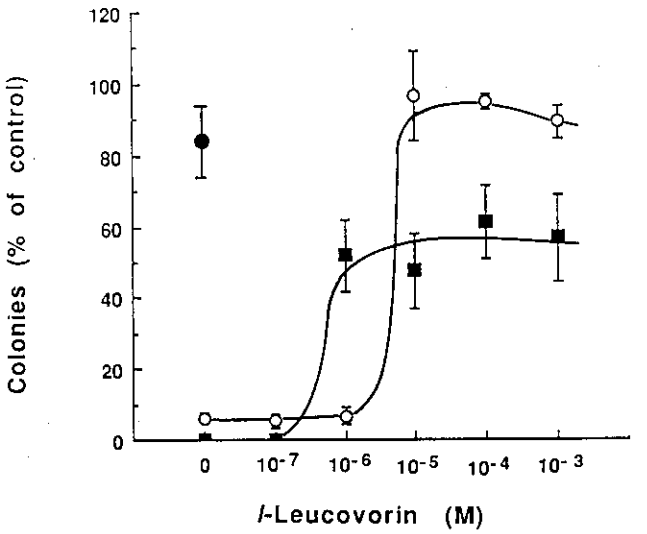

Fig. 3. Reversal of MTX cytotoxicity to MTX-sensitive K562 and -resistant K562/MTX cells by *l*-leucovorin. At the extracellular MTX levels of 10^{-6} *M* for K562 cells (■) and 10^{-5} *M* for K562/MTX (○), almost the same total intracellular MTX levels in both cell lines are obtained, as shown in Fig. 5. ●, 10^{-6} *M* MTX for K562/MTX cells. Each value is expressed as the mean±SD of 3 separate experiments.

DISCUSSION

The present study suggested that there may be a correlation between intracellular MTX-PG formation and the reversal of MTX cytotoxicity by *l*-LV. Until recently, it has been thought that the *l*-LV concentration required to rescue cells from cytotoxicity varies directly with MTX concentration, a relationship that has been explained in

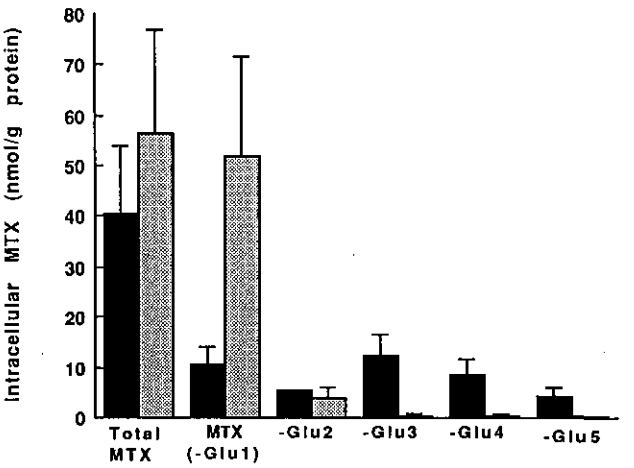

Fig. 5. Total intracellular MTX concentrations and MTX-polyglutamate profiles in K562/parent (■) and MTX-resistant K562/MTX cells (▨) after 24-h incubation with 10^{-6} *M* and 10^{-5} *M* MTX, respectively. Each value is expressed as the mean±SD of 5 separate experiments.

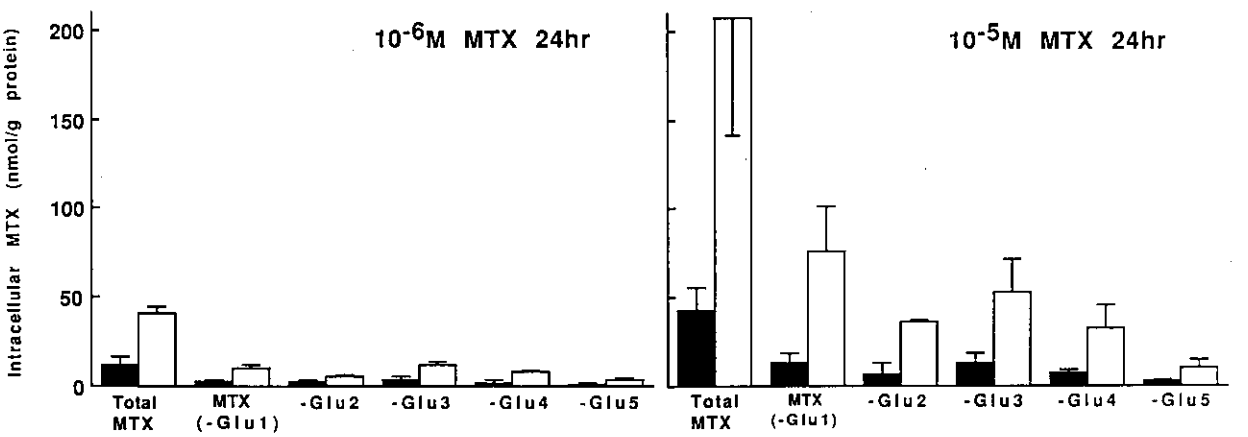

Fig. 4. Total intracellular MTX concentrations and MTX-polyglutamate profiles in purified bone marrow myeloid progenitor cells (■) and K562 cells (□) after incubation with 10^{-6} *M* and 10^{-5} *M* MTX for 24 h. Each value is expressed as the mean±SD of 5 separate experiments.

terms of competition for intracellular membrane transport.[18,20] A new possibility suggested by the present work is that an increase of MTX-PG formation could cause the failure of *l*-LV to overcome inhibition, through a noncompetitive mechanism.

One of the important pharmacologic features of MTX-PGs is preferential retention in cells even after the removal of extracellular MTX.[10,11] According to our previous data,[5,16] significant fractions of MTX(-Glu1) and MTX-Glu2 were rapidly lost from the cells. In contrast, longer MTX-PGs (MTX-Glu3–5) remained in the cells for at least 24 h after the removal of free extracellular drug and continued to exert an inhibitory effect on DHFR. Another important property of MTX-PGs is their increased binding affinity to folate-dependent enzymes. Comparative studies of the rates of binding of MTX and MTX-Glu5 to highly purified DHFR from human breast cancer cells have confirmed a 40-fold greater affinity of the latter for DHFR. Recent studies by Allegra *et al.* have shown that MTX-PGs have markedly enhanced inhibitory potency for other folate-dependent enzymes, including TS[13] and AICAR transformylase[14]; the latter is one of the key enzymes in the *de novo* purine synthetic pathway. Thus, it is likely that MTX-PGs directly inhibit purine and thymidylate synthesis. Dihydrofolate polyglutamates, which increase in the cell following inhibition of DHFR by MTX, have also been reported to enhance the inhibition of TS and AICAR transformylase.[14]

In our comparative studies using MTX-sensitive K562 and -resistant K562/MTX cells, much greater formation of MTX-PGs was observed in sensitive K562 cells, accompanied with lesser effectiveness of *l*-LV for the reversal of MTX cytotoxicity. These data suggested that significant amounts of MTX-PGs were formed in K562 cells during the initial 24-h incubation with MTX and completely blocked various enzymes, not only DHFR, but also TS and AICAR and probably glycinamide ribonucleotide (GAR) transformylases. It might be impossible to prevent MTX cytotoxicity to even such severely enzyme-blocked cells by using a very high concentration of *l*-LV. On the other hand, possibly because of diminished formation of MTX-PGs in K562/MTX cells, MTX cytotoxicity to these cells was effectively reversed after the addition of high concentrations of *l*-LV. The greater (more than equimolar) amount of *l*-LV required for BM G-CFCs with high concentrations (10^{-6} *M* or more) MTX was noted in mice[18] as well as in humans as described in this study. These findings may also be explained by increasing formation of MTX-PGs in BM myeloid progenitor cells cultured with increasing extracellular concentrations of MTX.

K562/MTX cells were cloned in our laboratory and the mechanisms of their drug resistance were studied as described previously.[16] In addition to impairment of MTX-PG formation and decreased membrane transport, K562/MTX cells also showed changes of the binding sites of DHFR for MTX without increase of DHFR activity and a reduction of TS activity (≈25% of control) (manuscript submitted for publication). Although the role of these latter factors, if any, in the reversal of MTX cytotoxicity by *l*-LV remains to be elucidated, changes of MTX-PG formation seem much more likely to be correlated to the reversal by *l*-LV because MTX-PGs act so potently on the folate-dependent enzymes.

The metabolic rate of MTX, including membrane transport and MTX-PG formation, is dose- and time-dependent, and is also suggested to be widely different among different tissues. The present study raises the possibility that, in addition to a competitive reversal by LV of MTX cytotoxicity through a common membrane transport system, a noncompetitive reversal relating to intracellular MTX-PG formation may be another important factor in the mechanism of the reversing ability of LV. Further studies will be needed to obtain direct evidence of this. The ideal use of LV involves the minimum recovery of tumor cell growth from MTX cytotoxicity and the maximum rescue of normal tissues. In this regard, these data may be useful in the clinical setting to design a new protocol including high-dose MTX/*l*-LV chemotherapy.

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