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High-dose methotrexate therapy with leucovorin rescue: in vitro investigations on human osteosarcoma cell lines*

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Summary. High-dose methotrexate (MTX) therapy with subsequent leucovorin (LV) rescue (HDMTX-LV) in the treatment of osteosarcoma is based on the assumption that this tumor has a deficient uptake system for MTX and reduced folates. To simulate features of HDMTX-LV therapy protocols in vitro , sensitive and MTX-resistant human osteosarcoma cell lines and a lymphoblastoid cell line were exposed to MTX and/or LV at various dosages and time schedules and the effects on DNA metabolism and on cell growth were evaluated. The data show that in osteosarcoma cells and in lymphoblasts the cytotoxic effects of 10^{-6} *M* to 10^{-7} *M* MTX can be substantially reversed by LV if the antidote is applied within the first 12 h of MTX exposure. The results are not consistent with the assumption mentioned above and should be taken into consideration when designing new therapeutic regimens. An alternative hypothesis for the efficacy of HDMTX-LV is discussed. It is concluded that HDMTX-LV therapy may be effective in the treatment of osteosarcoma, even when subpopulations of the tumor cells exhibit different mechanisms of resistance to MTX, such as elevated levels of dihydrofolate reductase or a deficient transport system for MTX, if high doses of MTX are applied long enough to ensure lethal intracellular MTX levels and low-dose LV schedules instituted after a long delay are used.

Introduction

MTX mediates its cytotoxic effects through inhibition of dihydrofolate reductase (DHFR), which results in a depletion of intracellular tetrahydrofolate pools and thus leads to a decline of de novo DNA synthesis and to a decreased incorporation of exogenous deoxyuridine (dUR) into DNA [14]. As a consequence of the subsequently contracted thymidine triphosphate pool, thymidine kinase activity is increased (salvage pathway) and incorporation of exogenous thymidine (dTR) into DNA is elevated [16]. Thus, the ratio of dUR to dTR incorporation into DNA can be used as a measure to determine the effects of MTX on DNA metabolism [11, 12]. LV (5-formyltetrahydrofolate) serves as a source of reduced folates to replenish cellular pools depleted by MTX action [11, 12, 14].

High-dose MTX therapy followed by LV rescue (HDMTX-LV) is used extensively in the clinical treatment of osteosarcoma as an adjuvant to surgery [7, 10]. Since this tumor was found not to respond to conventional low-dose MTX therapy, HDMTX-LV regimens have been evaluated. They were based on the assumption that in osteosarcoma cells the uptake system for reduced folates, which is known to be shared by MTX [8], is highly impaired [7, 12]. At high serum concentrations, MTX can enter the cells by passive diffusion and thus reach cytotoxic concentrations, whereas the antidote LV, applied at relatively low doses, is thought to be selectively accumulated in normal cells, which possess an effective transport system for reduced folates.

In the design of HDMTX-LV regimens, various empirically established dose schedules have been evaluated, but little information is available concerning the in vitro effects of MTX on human osteosarcoma cells [3]. Using conditions designed to simulate pharmacologically definable features of HDMTX-LV protocols in vitro, we investigated the effects of MTX on DNA metabolism and on cell survival, and also the potential of LV to overcome these effects in two osteosarcoma cell lines of human origin and in two MTX-resistant sublines exhibiting different mechanisms of resistance. To study HDMTX-LV effects on normal cells, a representative lymphoblastoid line, RPMI 1788, was employed.

Materials and methods

All chemicals and media were of the purest grade available from Biochrom, Berlin, Merck, Darmstadt, and Sigma, Munich. Methotrexate was obtained from Lederle, Wolfratshausen. Deoxy-[6-^{3}H]uridine (specific activity 0.55–1.1 TBq/mmol) and [methyl-^{3}H]thymidine (specific activity 1.5–2.2 TBq/mmol) were obtained from Amersham, Braunschweig. Quickszint 501 was obtained from Zinsser, Frankfurt.

Cell culture. All cells in this study are of human origin. The osteosarcoma cell lines TE-85 and SAOS-2 were kindly donated by Dr J. Fogh, Sloan-Kettering Institute, New York. The MTX-resistant sublines TE-85/MTX and SAOS-2/MTX were selected in vitro as described previ-

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ously [3]. TE-85/MTX has 19-fold resistance to MTX compared with the parental strain TE-85. Resistance to MTX is associated with a 5.5-fold overproduction of the target enzyme DHFR owing to a 6.6-fold amplification of the DHFR gene (unpublished results). These cells show normal initial uptake rates of MTX. The SAOS-2/MTX cell line exhibits a 200-fold resistance to MTX. MTX uptake in these cells is decreased to 32% compared with SAOS-2, whereas the level of DHFR is in the same range as in the parental strain. All osteosarcoma cells were propagated as monolayers in Eagle's MEM. The lymphoblastoid line RPMI 1788 (ATCC CCL 156) was obtained from the American Type Culture Collection, Rockville, Md. RPMI 1788 cells were grown in suspension culture in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum, penicillin G (100 units/ml) and streptomycin (100 µg/ml). Cultures were incubated at 37° C, 95% humidity, in a 5% CO_2 atmosphere. The uninhibited mean log-phase doubling times were: TE-85, 30 h; TE-85/MTX, 41 h; SAOS-2, 60 h; SAOS-2/MTX, 62 h; RPMI 1788, 25 h.

Incorporation of ^{3}H-dUR and ^{3}H-dTR into DNA. Exponentially growing cells were exposed to the appropriate concentrations of MTX and/or LV during the time intervals indicated. After removal of the drugs, the cells were incubated for 1 h at 37° C with ^{3}H-dUR or ^{3}H-dTR (both: 10^{-6} *M*, specific activity 14.8 GBq/mmol). The medium was removed and cells were lysed with 0.3 *N* NaOH for 15 min. An aliquot of 1 ml was chilled on ice. After addition of 1 ml 15% ice-cold trichloroacetic acid (TCA), the acid-insoluble material was precipitated on cellulose-acetate membrane filters (0.45 µm) by washing three times with 5 ml ice-cold 5% TCA and twice with 5 ml 0.9% NaCl. Filters were dried (20 min, 80° C), and counts were measured in 10 ml Quickscint 501 in a 1211 Rackbeta (LKB) liquid scintillation counter.

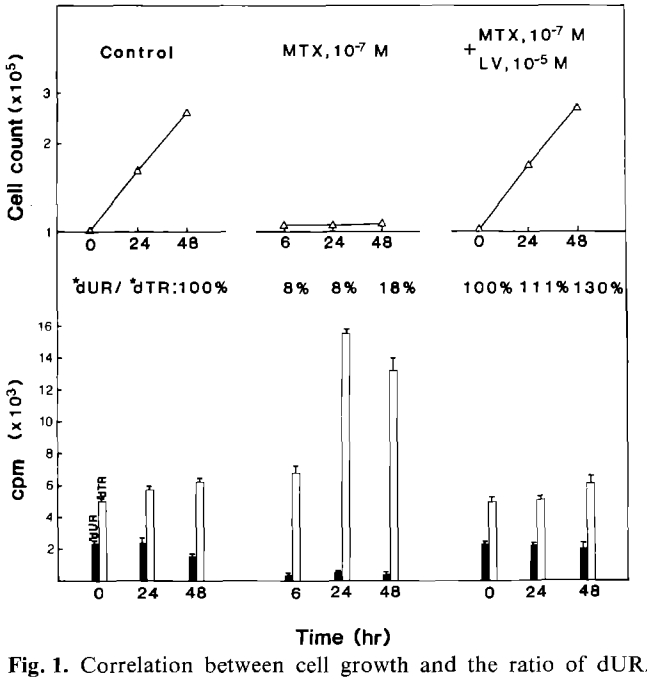

Fig. 1. Correlation between cell growth and the ratio of dUR/dTR incorporation into DNA of TE-85 osteosarcoma cells treated with MTX or a combination of MTX and LV. *Bars*, SD

Results

To evaluate whether changes in DNA metabolism reflect cytotoxic effects of MTX on osteosarcoma cells, the ratio of dUR/dTR incorporation into DNA of TE-85 cells was estimated and compared with cell survival rates (Fig. 1). Treatment with 10^{-7} *M* MTX resulted in cessation of cell growth, and dUR incorporation was almost completely blocked, whereas dTR incorporation increased considerably. The dUR/dTR quotient in these cells was reduced to 8% after 6 h and 24 h, and to 18% after 48 h of MTX exposure. Simultaneous application of 10^{-7} *M* MTX and 10^{-5} *M* LV resulted in normal cell growth and in dUR/dTR incorporation ratios that slightly exceeded the ratios found for controls without LV and MTX. Thus, in the subsequent assays on LV rescue, cells treated with the appropriate LV concentrations for the indicated time intervals served as controls.

To estimate the concentration ratio of LV to MTX necessary to compensate MTX cytotoxicity, TE-85 cells were exposed to various doses of MTX and LV, as indicated in Fig. 2a. The dUR/dTR quotient reflecting the effect of

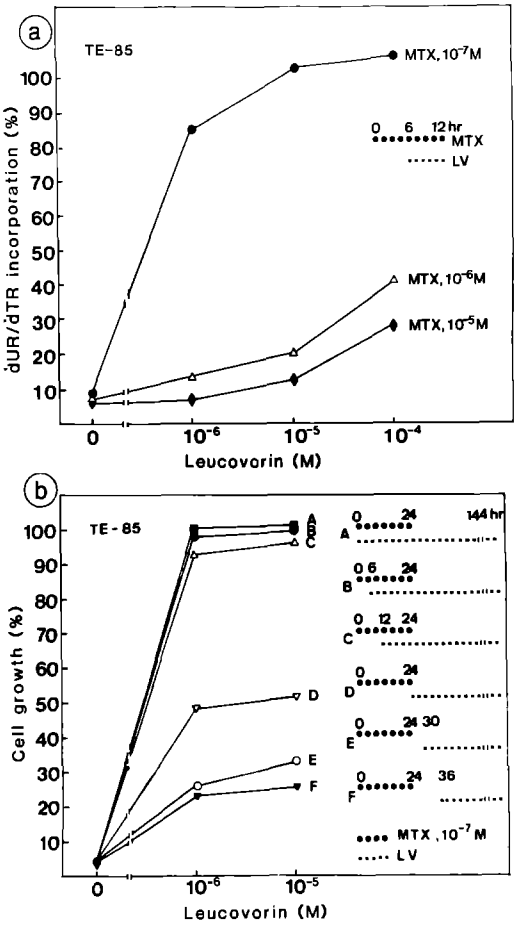

Fig. 2. a Effect of various doses of MTX and/or LV on DNA metabolism in TE-85 cells determined as dUR/dTR incorporation rates into DNA. MTX was present for 12 h, and LV was added 6 h after MTX application prior to 1 h pulse-labeling. b cell survival rates as percentages of controls after treatment of TE-85 cells with MTX (10^{-7} *M*) and various doses of LV. MTX was present for 24 h, and LV was added at the time intervals indicated. Cell count was done 6 days after initiation of cultures. Results are means of three separate experiments done in triplicate. SD was consistently < 10%

10^{-7} *M* MTX was elevated from 8% to 86% of controls by LV added at a concentration of 10^{-6} *M*, and increased amounts of LV resulted in a complete reversal of MTX toxicity. In cells treated with 10^{-6} *M* or 10^{-5} *M* MTX, the compensating effect of even 10^{-4} *M* LV was less pronounced, but still detectable. To explore the time-effect correlation of LV application in respect to MTX administration, TE-85 cells were exposed to MTX and/or LV at various doses and time schedules, based on therapeutic regimens used in HDMTX-LV, and cell survival rates were measured (Fig. 2b). When 10^{-6} *M* LV was added within the first 12 h after MTX application, this resulted in a complete reversal of MTX cytotoxicity (Fig. 2b, A–C). When LV application was delayed for 24 h (Fig. 2b, D) the effect was less extensive, but even 24 h after cessation of MTX treatment an enhancement of cell growth by LV from 4% to 21% could be observed.

According to the results obtained for TE-85, LV effectively normalized the dUR/dTR quotient in a second osteosarcoma line, SAOS-2, treated with 10^{-7} *M* MTX (Fig. 3a). The effect of 10^{-6} *M* MTX on DNA metabolism could still be substantially reversed (from 31% to 80%) by equimolar amounts of LV. Only the effect of 10^{-5} *M* MTX was normalized to a lesser extent. Investigation of cell survival rates in SAOS-2 cells revealed that LV, when applied in equimolar doses to MTX within the first 6 h, circumvented MTX cytotoxicity. At a later time point no significant enhancement of cell survival by LV was observed.

In addition to the osteosarcoma cell lines TE-85 and SAOS-2, two MTX-resistant sublines, TE-85/MTX and SAOS-2/MTX, each possessing a distinct mechanism of resistance to MTX were investigated. Resistance in TE-85 is based on an increased level of DHFR. As can be seen in Fig. 4a, the decrease in the dUR/dTR incorporation rate to 65% following exposure to 10^{-6} *M* MTX was completely reversed by equimolar amounts of LV. In contrast to the results found for the sensitive cell line TE-85, the impact on DNA metabolism of 10^{-5} *M* MTX in TE-85/MTX cells was markedly reversed by 10^{-5} *M* LV. Even at very high MTX concentrations (10^{-4} *M*), a significant increase in dUR/dTR incorporation rates by equimolar LV concentrations was noted. The results of growth-inhibition experiments, summarized in Fig. 4b, show that LV added in equimolar amounts to TE-85/MTX cells treated with 10^{-5} *M* MTX could reverse MTX cytotoxicity when applied within the first 6 h after MTX. When addition of LV

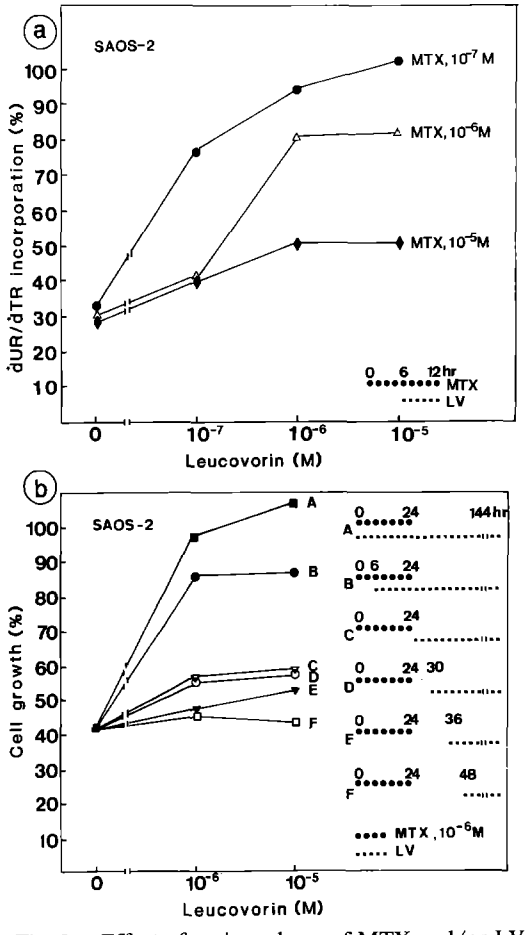

Fig. 3. a Effect of various doses of MTX and/or LV on DNA metabolism in SAOS-2 cells determined as dUR/dTR incorporation rates into DNA. **b** Cell survival rates as percentages of controls after treatment of SAOS-2 cells with MTX (10^{-6} *M*) and various doses of LV. MTX was present for 24 h, and LV was added at the time intervals indicated. Results are means of three separate experiments done in triplicate. SD was consistently < 10%

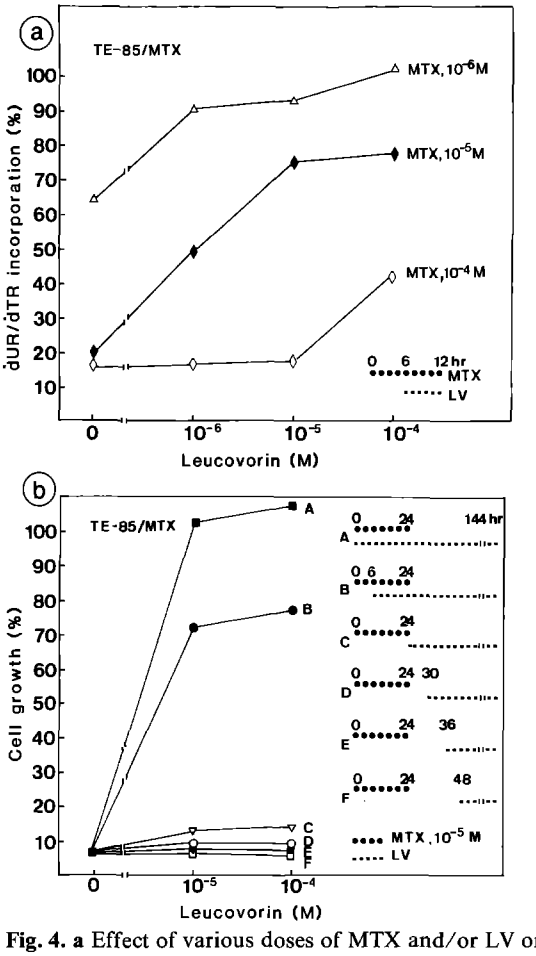

Fig. 4. a Effect of various doses of MTX and/or LV on DNA metabolism in MTX-resistant TE-85/MTX cells determined as dUR/dTR incorporation rates into DNA. **b** Cell survival rates as percentages of controls after treatment of TE-85/MTX cells with MTX (10^{-5} *M*) and various doses of LV. MTX was present for 24 h, and LV was added at the time intervals indicated. Results are means of three separate experiments done in triplicate. SD was consistently < 10%

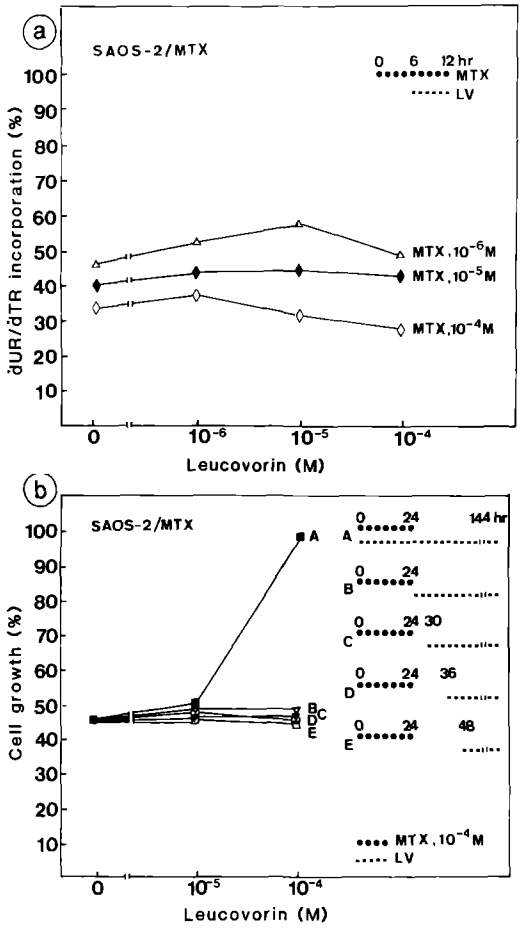

Fig. 5. a Effect of various doses of MTX and/or LV on DNA metabolism in MTX-resistant SAOS-2/MTX cells determined as dUR/dTR incorporation rates into DNA. b Cell survival rates as percentages of controls after treatment of SAOS-2/MTX cells with MTX (10^{-4} *M*) and various doses of LV. MTX was present for 24 h and LV was added at the time intervals indicated. Results are means of three separate experiments done in triplicate. SD was consistently < 10%

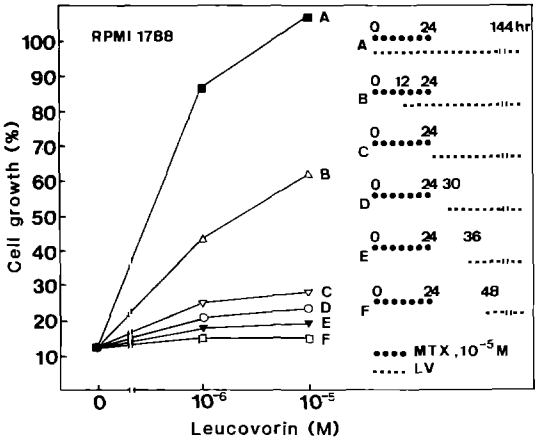

Fig. 6. Cell survival rates as percentages of controls after treatment of RPMI 1788 cells with MTX (10^{-5} *M*) and various doses of LV. MTX was present for 24 h, and LV was added at the time intervals indicated. Results are means of three separate experiments done in triplicate. SD was consistently < 10%

was delayed for 24 h or more no enhancement of cell survival could be observed.

In SAOS-2/MTX cells the resistance is based on a decreased membrane transport of MTX. The data in Fig. 5a show that even high doses of LV fail to diminish the MTX effect in these cells. Accordingly, as can be seen from Fig. 5b, no other protocol except simultaneous application of 10^{-4} *M* LV enhanced cell survival in SAOS-2/MTX treated with 10^{-4} *M* MTX.

To compare the results obtained with malignant osteosarcoma cells with normal hematopoietic cells, the response of the lymphoblastoid cell line RPMI 1788 to MTX and LV was investigated. RPMI 1788 cells revealed a high intrinsic resistance to MTX. Nonetheless, as depicted in Fig. 6, the cytotoxic effect of high concentrations of MTX (10^{-5} *M*) was reversed by relatively low (10^{-6} *M*) concentrations of simultaneously added LV. The enhancement of cell survival was less marked when LV application was delayed for 12 h (Fig. 6, B), and only a marginal degree of rescue could be observed when LV addition was delayed for 24 h or more (Fig. 6, C–F).

Discussion

The in vitro studies described in this paper were initiated in an attempt to provide a conceptual basis for further optimization of HDMTX-LV protocols that may be useful at the clinical level. Simulating in vivo conditions of HDMTX-LV regimens, the MTX and LV concentrations used were equivalent to serum levels attained clinically during the first 48 h after cessation of i.v. MTX administration [1, 5, 12, 14]. The antimetabolic effect of several MTX/LV treatment protocols was measured in osteosarcoma cell lines of human origin and in osteosarcoma cells with different defined mechanisms of resistance to MTX, and the results were compared with those obtained in a lymphoblastoid cell line. The data show that a resumption of DNA synthesis induced by LV did occur in MTX-sensitive osteosarcoma cells pretreated with 10^{-7} *M* MTX, this concentration reflecting the in vivo plasma level 48 h after cessation of MTX application. The effect on DNA metabolism of higher amounts of MTX (10^{-6} *M*, 10^{-5} *M*), clinically achieved as plasma levels within the first 24 h after MTX application, was only partially reversed even by high concentrations of LV (10^{-5} *M*, 10^{-4} *M*).

In contrast to the results obtained with sensitive TE-85 cells, an extensive normalization of the effect of 10^{-5} *M* MTX on DNA metabolism by LV (10^{-5} *M*, 10^{-4} *M*) was noted in the MTX-resistant TE-85/MTX cell line, which is known to have elevated DHFR levels [3]. This result seems to imply a limited effectiveness of HDMTX-LV regimens if tumor cells in patients exhibit this mechanism of resistance. In experiments on cell survival, however, where cells were exposed to MTX and/or LV under conditions more closely reflecting the in situ situation concerning the time schedules applied, no rescue could be observed when LV application was delayed for 24 h or more after MTX treatment. Under the conditions described in this paper, the ratio of dUR/dTR incorporation rates is informative for DNA metabolism after 12 h of MTX/LV treatment, but does not reflect additional factors that may influence LV rescue under long-term MTX exposure (24 h).

In a previous study, the data indicated that MTX was transported into osteosarcoma cells by a carrier-mediated

uptake system [3]. Resistance to MTX in the osteosarcoma line SAOS-2/MTX is based on a decrease in uptake of reduced folates, thus representing properties that have been assumed to underlie the effectiveness of HDMTX-LV regimens. As expected, in this cell line MTX toxicity could not be overcome by LV unless extremely high concentrations (10^{-4} *M*) of the antidote were applied simultaneously with MTX treatment.

Since in HDMTX-LV therapy bone marrow toxicity is the limiting factor, the hematopoietic cell line RPMI 1788 was included in the investigations on LV rescue protocols. The cell line is originated from a normal male; it has not been intentionally transformed, and EBV has not been detected [6]. RPMI 1788 cannot definitely be considered a normal cell line; concerning the response to MTX, however, these cells closely resemble normal hematopoietic cells. The observed high intrinsic resistance to MTX is in accordance with previous reports that MTX sensitivity is lower in normal cells than in tumor cells [2, 14]. The data show that LV application, when delayed for 24 h or more, did not enhance cell survival of RPMI 1788 cells. The discrepancy concerning proliferation characteristics of these cells under in vitro conditions compared with the actual in vivo situation may explain this phenomenon. In the assay described in this paper most cells pass the S-phase during the 24 h of MTX exposure. In the bone marrow, however, only 10%–20% of blasts are actually proliferating [15], and they are thus less susceptible to the action of MTX. In contrast, tumor cells, especially those in mitotically active metastases, will be more sensitive to MTX [15].

It is interesting to note that the amount of LV needed for reversal of the cytotoxic effects of MTX increases more than proportionately with respect to the concentration of MTX used. This suggests that an additional mechanism may be responsible for the disproportionate increase in MTX toxicity when it is used in high concentrations over a long time. MTX, like physiologic folates, can be metabolized to γ-polyglutamate derivatives, which exhibit high affinity for DHFR but low affinity for the efflux system of folates and therefore are capable of causing delayed cytotoxicity [7, 13]. Since polyglutamate synthesis is favored by high levels of MTX and by long exposure to the drug [9], we therefore suggest that this metabolic feature may account for the above-mentioned effect. Further, when tumor cells are exposed to the cytotoxic action of MTX for an extended period due to polyglutamate formation, lower dosages of LV will promote recovery in normal cells more effectively than in tumor cells, since the former are known to have lower polyglutamate synthetase activity [4, 9]. Thus, formation of MTX polyglutamates may be the basis for the efficacy of HDMTX-LV therapy [13].

In accordance with the work of Sauer et al. [11, 12], who investigated dUR/dTR incorporation rates in bone marrow cells from patients receiving HDMTX-LV, our data show that effective LV rescue cannot be expected until the plasma MTX concentration falls below 10^{-6} *M*. The results indicate, however, that if LV is given either too early (within the first 24 h) or in excess the tumor may be spared from MTX cytotoxicity just as normal tissue is.

In conclusion, the hypothesis that HDMTX-LV therapy is effective in osteosarcoma because of a deficient transport system for LV in comparison with normal cells seems to be incorrect. The data presented here indicate that HDMTX-LV may be effective in therapy of osteosarcoma even if subpopulations of the tumor cells exhibit different mechanisms of acquired resistance to MTX, provided that high doses of MTX are applied for long enough to ensure high intracellular MTX levels and subsequent polyglutamate formation in tumor cells, and that low-dose LV schedules instituted after a long delay are used.

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