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The Biochemistry of the Citrovorum Factor Rescue Effect in Normal Bone Marrow Cells After High-Dose Methotrexate*

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Abstract—*Incorporation rates of* ^{3}H-*deoxiuridine and* ^{3}H-*thymidine into the DNA of bone marrow cells and thymidine kinase activity in these cells were compared with the methotrexate serum concentrations in two patients with osteogenic sarcoma under high dose methotrexate therapy with following citrovorum factor administration (CF-rescue). Methotrexate decreases the ratio of deoxiuridine versus thymidine incorporation and increases thymidine kinase activity. Using these biochemical parameters it could be shown that the usually administered citrovorum factor doses (about* 100 mg *per* day) *are not sufficient for an effective rescue for the bone marrow cells as long as serum methotrexate concentrations are equal or higher than* 10^{-6} M. *In critical cases with retarded methotrexate elimination the monitoring of the DNA metabolism of the bone marrow cells can determine whether the used citrovorum factor dose is really enough to preserve the patients from severe methotrexate toxicity.*

INTRODUCTION

HIGH-DOSE methotrexate therapy with following citrovorum factor rescue (HDMTX-CF) is successfully used in the adjuvant chemotherapy [1–4] and in the treatment of metastatic osteosarcoma [1–7]. The adjuvant chemotherapy produces a 5 yr survival rate of 82% [4]. Sequential combination of HDMTX-CF and adriamycin seems to give better results with longer duration of disease free survival and of remission, respectively [8]. Favourable results of HDMTX-CF are reported in a variety of neoplastic diseases [9–15], controlled studies comparing this therapy with other chemotherapeutic regimens are still lacking, however.

HDMTX-CF is based on the assumption that primary resistant tumours have an impaired active membrane transport system for folate compounds which is normally shared by methotrexate (MTX). However, at very high serum concentrations MTX can enter these cells by passive diffusion, independent of the active transport system resulting in cytocidal intracellular MTX concentrations [16–19]. Normal rapidly proliferating tissues with an intact active transport system for folates can be protected from death by MTX by the application of relatively small doses of the antidote citrovorum factor (CF = Leucovorin™). This is the so-called rescue effect of CF. Since essentially no CF can enter the cells by passive diffusion at low serum concentrations, tumour cells lacking the active transport systems do not benefit from the rescue effect.

Methotrexate specifically inhibits the dihydrofolate reductase and stops the reduction of FH_2 to FH_4 and consecutively diminishes the pool of activated one-carbon (C_1) units. Thus,

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Abbreviations: CF = citrovorum factor = 5-formyl-tetrahydrofolic acid = CHO–FH_4 = Leucovorin™; DNA = deoxyribonucleic acid; dTR = thymidine; dTMP = thymidine monophosphate; dTTP = thymidine triphosphate; dUR = deoxyuridine; dUMP = deoxyuridine monophosphate; FH_2 = dihydrofolic acid; FH_4 = tetrahydrofolic acid; MTX = methotrexate; HDMTX = high-dose methotrexate treatment.

a substrate deficiency for thymidylate synthetase leads to a decline of the *de novo* synthesis of dTMP (from dUMP and 5-10-methylene-FH_4) which results in a decreased incorporation of exogenous ^{3}H-dUR into the DNA [15, 19–21]. The diminished dTTP-production releases the normal feedback inhibition of the thymidine kinase. The incorporation of exogenous ^{3}H-dTR into the DNA increases (salvage pathway) [21, 22]. Normally the incorporation rates of dUR and dTR are almost equal. The normal range of the ratio dUR/dTR is 0.7–1.3 [23]. In the presence of high MTX concentrations this value is less than 0.1.

These biochemical effects of MTX can be corrected by the administration of its antidote CF since this tetrahydrofolate compound enters directly the pool of the activated C_1-units [24]. Thus, dTMP synthesis and *de novo* purine synthesis can resume although the dihydrofolate reductase is still inhibited by MTX. The rescue effect of CF is indicated by the normalization of the dUR/dTR ratio. There are many biochemical investigations on the CF-rescue in tumour bearing mice [25] but only a few in humans. In humans dUR incorporation rates of normal bone marrow cells were measured after *in vitro* exposure to 10^{-6} M MTX [26]. The inhibition of dUR incorporation under HDMTX-CF was estimated in peripheral blasts of leukaemic patients [27], and in bone marrow aspirates of 4 patients with different tumours [28]. Yet serum concentrations of MTX were not measured. Therefore, it was not possible to correlate the actual MTX levels with its biochemical effects [28]. *In vitro* it was estimated that a 10-fold CF concentration was necessary for the reversal of toxicity of 10^{-6} M MTX on bone marrow cells [15]. Thus, until now it is not clear at which time after MTX and at which MTX concentration CF rescue is really effective for normal bone marrow cells *in vivo* when given in conventional doses. By simultaneous measuring of serum MTX concentrations and of dUR and dTR incorporation rates into the DNA of bone marrow cells we intended to answer these questions.

MATERIALS AND METHODS

1. *Permanent lymphoblast culture LS_2*

Derived from a patient with acute undifferentiated leukaemia, until now more than 150 passages. Culture medium (all substrates from Flow-Lab.): RPMI 1640-medium with glutamine (3 mM), penicillin/streptomycin (100 i.u./100 ng/ml) and 20% foetal bovine serum (30 min inactivated at 56°C). Cultures at 37°C, water vapour saturated atmosphere with 5% CO_2 (Heraeus incubator KB 600 CO_2).

2. *Isolation of bone marrow cells*

Immediately after the puncture a 2 ml aliquot of bone marrow is added to 2 ml of buffered Ringer–phosphate–glucose solution with 1% Mg–EDTA (10 ml 1/15 M potassium phosphate buffer pH 7.4 mixed with 90 ml Ringer solution; Ringer solution consits of 6.2 g NaCl + 0.2 g KCl + 0.1 g $MgCl_2$ + 2.0 g $NaHCO_3$ + 2.0 g glucose + 10 g Mg EDTA + bi-distilled water to 1000 ml). Erythrocytes are lysed at 0°C in 5 ml 0.84% ammonium chloride (3 min). After 3-times washing with the buffered Ringer solution (now without EDTA) one obtains an erythrocyte free fraction of nucleated bone marrow cells [22].

3. *Preparation of enzyme solution from bone marrow cells*

The suspension of nucleated cells (see 2.) is centrifuged (5 min, 800 ***g***, 4°C). 10^5 cells/mm^3 are suspended in 0.001 M potassium phosphate buffer pH 7.4 and lysed by incubation for 1 hr at 0°C. After centrifugation (20 min, 50,000 ***g*** 4°C) enzyme activities are measured in the clear supernatant [22].

4. *Incorporation of thymidine (dTR) and deoxyuridine (dUR) into the DNA of intact cells*

(*a*) *Lymphoblast culture.* Five-hundred microlitres of the cell suspension (about 10^6 cells) are incubated for 1 hr with 10^{-6} M ^{3}H-dTR (specific activity 0.5 Ci/mmol; Amersham, TRA 306) or 10^{-6} M ^{3}H-dUR (0.5 Ci/mmol; Amersham, TKR 242). After chilling in ice, the acid insoluble material is precipitated on cellulose–acetate membrane filters (Millipore, HAWP 02500, 0.45 μm) by washing the cell suspension on the filter 3 times with 2 ml of 5% trichloroacetic acid and 3 times with 2 ml of 0.1 N HCl. The filters are dried (20 min, 80°C) and their radioactivity is measured in 10 ml of an Omnifluor/toluene solution (5 g Omnifluor per 1000 ml of toluene) in a liquid scintillation counter.

(*b*) *Bone marrow cells.* (2×10^6) Isolated cells (see 2.) are incubated for 2 hr in 1 ml of Eagle's minimal medium with 5% horse serum and 0.25% glutamine (all from Difco)

with the addition of 10^{-6}M ^{3}H-dTR or 10^{-6} M ^{3}H-dUR (see above). The further steps are identical to 4 (a) [20].

5. *Estimation of thymidine kinase activity*

One-hundred microlitres of the enzyme solution (see 3.) are incubated at 37°C for 1 hr together with 100 µl of the following substrate mixture: 0.2 M Tris-buffer pH 8.0 + 0.02 M $MgCl_2$ + 9×10^{-3} M ATP (Boehringer 15028) + 2.8×10^{-3} M ^{3}H-dTR (specific activity 0.7 Ci/mmol; Amersham, TRA 306). The reaction is stopped by boiling for 2 min. After a sharp centrifugation (2 min, Eppendorf-centrifuge) 20 µl aliquots of the supernatant are given on DEAE–cellulose discs (Whatmanpaper DE 81). The discs are washed for 10 min with 0.001 M ammonium formate, once for 5 min with distilled water, and once for 5 min with absolute alcohol. After drying (20 min, 80°C) the radioactivity on the discs is determined in 10 ml Omnifluor/toluene by means of scintillation counting [29].

6. *Assay of methotrexate serum concentrations*

The MTX serum level is determined by its inhibition of partially purified dihydrofolate reductase. The enzyme is enriched from mouse liver or from *Lactobacillus casei* by ammonium sulphate precipitation and affinity chromatography.

The activity of the dihydrofolate reductase is measured by the decrease of the light absorption at 340 nm when FH_2 is reduced to FH_4 and when NADPH is oxidized to $NADP^+$. To establish a calibration curve, different amounts of MTX are added to the patient's own serum *in vitro*. Aliquots of this serum are added to the dihydrofolate reductase assay. The dihydrofolate reductase activities with the MTX-containing serum are compared to an assay with the patient's MTX-free serum. The MTX-induced enzyme inhibition is proportional to the serum concentration of MTX. Thus, unknown MTX serum concentrations can be read from the calibration curve by estimation of the inhibition of the dihydrofolate reductase by this serum. A detailed description of this quick assay for MTX is in preparation [30].

7. *HDMTX/CF treatment*

Two milligrams of vincristine are injected i.v. into the patients. Half an hour later the MTX application is started and the total dose is infused permanently over a period of 6 hr. Two hours later CF is given i.v. in a dose of 15 mg every 3 hr (8 times) and then every 6 hr (8 times). This treatment is repeated every 2 weeks. During the first 3 applications the MTX dose is increased from 3 to 6 and to 7.5 g/m^2. Then it is held at the latter dose for the following courses. The MTX serum concentration is determined directly at the end of the MTX infusion and later on three times every 24 hr. At these times also bone marrow is aspirated for the examination of the DNA-metabolism. Normal renal function is a most important precondition for HDMTX/CF therapy. Moreover, the urine must be alkalized and a high fluid intake must be guaranteed. For detailed instructions see [15].

8. *Patients*

Both patients had had an amputation because of osteosarcoma of the left lower leg, and the right thigh, respectively. The first (47 yr) received HDMTX-CF when pulmonary metastases appeared 9 months after amputation, the second (22 yr) received HDMTX-CF as adjuvant chemotherapy. He tolerated the previous 5 cycles well without any sign of MTX toxicity.

RESULTS

The principles of the metabolic changes which are characteristic for the effects of MTX and CF were worked out with the model system of the permanent growing lymphoblast culture LS_2. These results are shown in Fig. 1.

In comparison to the control, 5-formyltetrahydrofolate (= citrovorum factor = CF) alone has no significant effect either on the cell growth or on the incorporation rates of ^{3}H-dTR or ^{3}H-dUR. Under normal conditions the incorporation rates for these two nucleosides are nearly equal, the quotient dUR/dTR lies within the normal range of 0.7–1.3. As expected from the biochemical mechanism, the dUR incorporation falls almost to zero under MTX whereas the dTR incorporation increases considerably. As a sign of a marked MTX effect the dUR/dTR quotient falls below 0.1. The DNA-metabolism of the cells is so much disturbed that reproduction can no longer occur. After 4 hr preincubation with 10^{-7} M MTX the addition of CF normalizes the incorporation rates. The quotient dUR/dTR raises again to the normal value of 1.3, culture growth is now regular again.

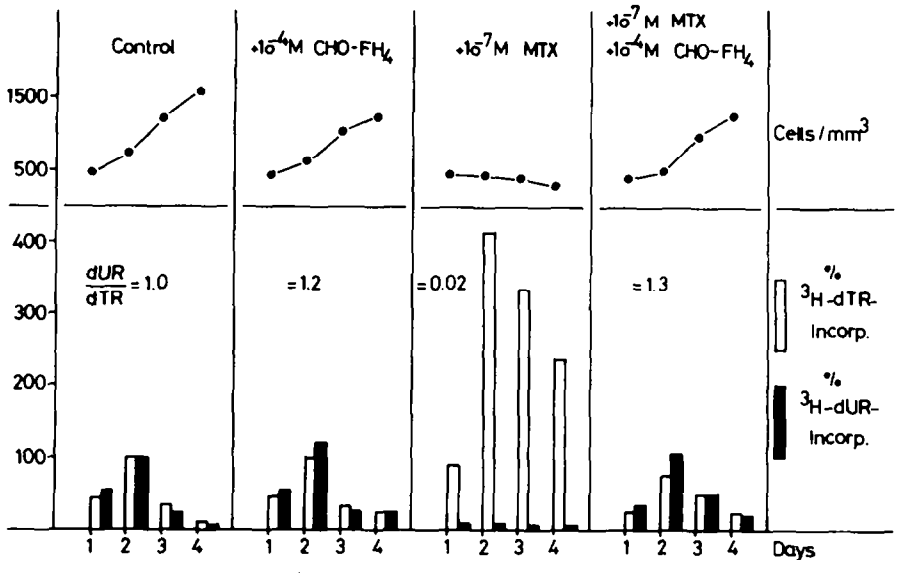

Fig. 1. DNA-metabolism in cultured human lymphoblasts (LS_2). Effects of methotrexate (MTX) and citrovorum factor (Leucovorin™ = CHO-FH_4).

The same parameters as in the model culture were estimated in bone marrow cells of patients under MTX therapy. Figure 2 shows the results of the dUR/dTR quotient and the corresponding MTX serum levels in a patient with metastatic osteosarcoma who received 3.8 and 14.0 g of MTX respectively. Even 24 hr after the end of the MTX infusion in both cases the DNA-metobolism in the bone marrow cells shows a clear MTX effect although up to that time already 7 doses of CF (15 mg each) have been injected. At 24 hr the values of the dUR/dTR quotients are 0.14 and 0.25 and so considerably below the normal range. Only after 48 hr, when the MTX serum concentration decreased to the region of 10^{-7} M, have the dUR/dTR quotients (0.81 and 0.90 respectively) and DNA-metabolism returned to normal.

A different course of another patient is demonstrated in Fig. 3. As expected, the dUR/dTR quotient after MTX application falls to 0.06. Although CF is given regularly until the 48th hr there is no significant increase in the dUR/dTR quotient. For unknown reasons the MTX elimination from the patient's serum is considerably retarded. On the third day after the MTX infusion the MTX concentration in the serum is still 10^{-5} M and even on the 11th day a pharmacologically effective level of 2×10^{-7} M is noted. At that time measurement of DNA-metabolism was impossible because of a totally empty bone marrow. On the 17th day the bone marrow regenerated. Now under continued CF administration at a serum MTX concentration of 3×10^{-8} M, the dUR/dTR quotient is again within the normal range. In spite of retake and continuation

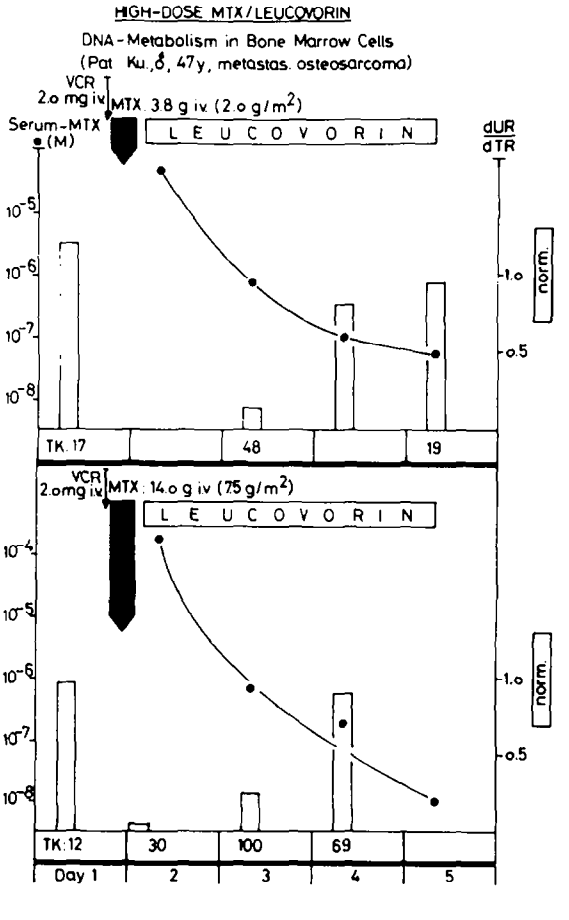

Fig. 2. MTX-serum concentrations and DNA-metabolism in bone marrow cells of a patient under HDMTX/CF therapy.

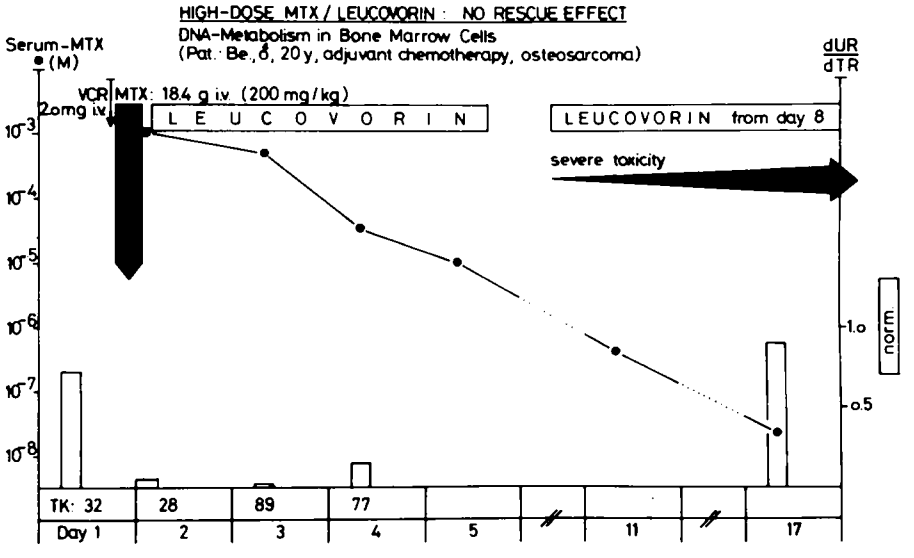

Fig. 3. Impaired MTX-elimination and toxic reaction under HDMTX/CF therapy. No biochemical rescue for the bone marrow.

of CF treatment at the first signs of toxic side effects (exanthema, leucocytopenia, thrombocytopenia) the patient developed a most severe bone marrow depression, and despite of intensive supportive care (antibiotics, thrombocyte- and granulocyte-transfusions) he died due to sepsis and renal insufficiency.

Another 8 patients were treated with 50 courses of HDMTX/CF without any complications.

DISCUSSION

The decrease of the quotient of dUR and dTR incorporation into the DNA of the cells is a good biochemical parameter for estimating the MTX effect on rapidly proliferating cell systems as lymphoblast cultures and bone marrow. The compensation of the MTX effect on the DNA metabolism by CF leads to the normalization of the dUR/dTR quotient (="rescue" effect of CF). Krishan *et al.* also measured the incorporation of dUR and dTR into bone marrow cells after HDMTX-CF. Their incorporation rates were changing considerably from day to day. As serum MTX concentrations were not measured, it could not be decided why these differences occurred and at which MTX levels CF rescue in these patients was effective [28]. Now, our data show that the usually administered doses of about 100 mg CF per day are not sufficient for an effective rescue for the bone marrow cells as long as the MTX concentration in the serum is equal or higher than 10^{-6} M. These results are in accordance to those of Bertino [15], who showed *in vitro* that a 10-fold higher CF concentration is necessary to reverse the toxicity of 10^{-6} M MTX.

At MTX serum concentrations of 10^{-7} M CF is effective in these doses and leads to the normalization of the dUR/dTR quotient. According to these results, early CF application before the 24th or 36th hr after the MTX infusion is not effective and seems to be of no benefit although this is demanded in most of the published schedules for HDMTX/CF therapy. In case MTX elimination is retarded and MTX serum levels remain above 10^{-6} M for more than 48 hr, the usually recommended CF doses are not sufficient to produce a significant rescue effect. In these patients CF rescue with "normal" doses is ineffective since both MTX and CF enter the cell by the same active transport mechanism and inhibit one another competitively [31]. Thus high MTX concentrations nearly totally prevent the transport of low CF doses into the cell. To save these patients from perhaps lethal damage of the bone marrow and/or the intestinal mucosa, the CF dose must be increased so much that the serum concentration of CF exceeds that of the MTX. For this purpose several grams of CF per day are necessary [32]. In critical cases the monitoring of the DNA metabolism in the bone marrow cells by means of the dUR/dTR quotient can determine if the used CF dose is enough to prompt the desired rescue effect.

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