Accumulation of Methotrexate Polyglutamates in Lymphoblasts Is a Determinant of Antileukemic Effects In Vivo

A Rationale for High-Dose Methotrexate

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Abstract

Methotrexate (MTX) is one of the most widely used drugs for the treatment of childhood acute lymphoblastic leukemia (ALL) and is commonly given in high doses. However, the rationale for high-dose MTX (HDMTX) has been challenged recently. To determine whether higher MTX polyglutamate (MTXPG) concentrations in ALL blasts translate into greater antileukemic effects, 150 children with newly diagnosed ALL were randomized to initial treatment with either HDMTX (1,000 mg/m^2 intravenously over 24 h) or lower-dose MTX (30 mg/m^2 by mouth every 6 h $\times$ 6). ALL blasts accumulated higher concentrations of MTXPG and long-chain MTXPG ($MTXPG_{LC}$) after HDMTX ($P < 0.00001$). Of 101 patients evaluable for peripheral blast cytoreduction, MTXPG concentrations were higher in patients whose blast count decreased within 24 h ($P = 0.005$) and in those who had no detectable circulating blasts within 4 days ($P = 0.004$). The extent of inhibition of de novo purine synthesis in ALL blasts was significantly related to the blast concentration of $MTXPG_{LC}$ ($IC_{95\%}$ = 483 pmol/10^9 blasts). The percentage of patients with 44-h $MTXPG_{LC}$ exceeding the $IC_{95\%}$ was greater after HDMTX (81%) than LDMTX (46%, $P < 0.0001$). These data indicate that higher blast concentrations of MTXPG are associated with greater antileukemic effects, establishing a strong rationale for HDMTX in the treatment of childhood ALL. (*J. Clin. Invest.* 1996. 97:73–80.) Key words: leukemia • methotrexate • pharmacodynamics • purine synthesis

Introduction

Acute lymphoblastic leukemia (ALL)[1] is the most common malignancy of childhood, and despite cure rates exceeding 70% (1–3) it remains one of the leading causes of death among children with cancer (1, 4). For these reasons, the development and refinement of more effective therapy for childhood ALL continues to be an important challenge. Methotrexate (MTX) is one of the most active agents for the treatment of ALL, and as such is a component of every modern ALL treatment regimen worldwide (5–10). However, despite four decades of use, the optimal dose of MTX remains controversial, with dosages ranging from 20 to 8,000 mg/m^2 in various ALL treatment protocols (6–10). Although one randomized clinical trial indicated that patients treated initially with high-dose methotrexate (HDMTX) had significantly better event-free survival compared with patients treated with lower-dose methotrexate (LDMTX) (6), questions have been raised about the rationale of using HDMTX in the treatment of childhood ALL (11, 12). It has been postulated that higher extracellular MTX concentrations do not necessarily produce higher intracellular concentrations in ALL blasts, due to saturation of receptor-mediated MTX uptake and saturation of metabolism to active MTX polyglutamylated metabolites (13–15). To address these concerns, we previously conducted a randomized study of HDMTX versus LDMTX as initial single-agent treatment of childhood ALL and established that significantly higher concentrations of methotrexate's active polyglutamylated metabolites (MTXPG) are achieved in leukemic blasts of patients treated with HDMTX compared with LDMTX (16). Although the achievement of higher concentrations of active MTXPGs in the target tissues provides a rationale for HDMTX in treating ALL, it is not known whether these higher intracellular concentrations translate into greater antileukemic effects. This investigation was therefore undertaken to extend our previous findings by assessing MTX's biochemical and cytotoxic effects on ALL blasts in children with newly diagnosed ALL, randomized to receive either HDMTX or LDMTX as the only antileukemic therapy during the initial 96 h after diagnosis.

Methods

Materials. MTX-glu_2 to MTX-glu_7 were purchased from Dr. B. Schircks (Jona, Switzerland); MTX radioenzymatic assay reagents were from The Enzyme Center Inc. (Malden, MA); [^{14}C]formate and formate were from ICN Biomedicals, Inc. (Costa Mesa, CA); RPMI 1640 and L-glutamine were from BioWhittaker, Inc. (Walkersville, MD); dialyzed and undialyzed fetal bovine serum were from Hyclone Laboratories (Logan, UT); adenine, guanine, and sodium acetate were from Sigma Immunochemicals (St. Louis, MO); acetonitrile was from Baxter (McGaw Park, IL); C18 Spherisorb HPLC column was from Phenomenex Inc. (Torrance, CA); Hepes and Hanks' were from Gibco Laboratories (Grand Island, NY); and heparin was from Fujisawa USA (Deerfield, IL). Intravenous HDMTX was prepared

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1. *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; AUC, area under the plasma concentration–time curve; Cp_{ss}, steady state plasma concentration; DI, DNA index; DNPS, de novo purine synthesis; HDMTX, high-dose MTX; LDMTX, lower-dose MTX; MTX, methotrexate; MTXPG, MTX polyglutamates.

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from MTX (Lederle Parenterals Inc., Cardina, PR), oral LDMTX was given as MTX tablets (Lederle Laboratories Division, Pearl River, NY).

Patients. Between December 1991 and August 1994, all patients 18 yr and younger with newly diagnosed ALL, excluding those with mature B cell phenotype, were enrolled on study protocol TOTAL XIIIA. Signed informed consent was obtained from parents or legal guardians before enrollment on the protocol. The diagnosis of ALL was based on morphology, cytochemical staining properties, and immunophenotyping of blast cells. The diagnosis of ALL was further subclassified as T-lineage (cytoplasmic $CD3^+$, $CD7^+$, plus $CD2^+$ or $CD5^+$ or both) or B-lineage (cytoplasmic $CD22^+$, $CD19^+$, $HLA\text{-}DR^+$, $CD10^{\pm}$), as previously described (17). Flow cytometric analysis was performed to determine the percentage of leukemic blast cells in S-phase and to determine ploidy based on the DNA index (DI; ratio of DNA content in leukemic G0/G1 cells versus normal diploid G0/G1 cells) (18).

Study protocol. All studies were conducted during the initial 96 h after diagnosis, before initiation of conventional remission induction therapy, as described previously in detail (16). Once enrolled, patients were stratified by age at diagnosis ($\leq$ 5 vs. $>$ 5 yr), DI ($<$ 1.16 or $>$ 1.6 vs. 1.16–1.6), and WBC count ($<$ 25,000 vs. $\geq$ 25,000), and randomized to receive either HDMTX or fractionated LDMTX as the only antileukemic agent during the initial 96 h after diagnosis. HDMTX was given as a 200 mg/m^2 intravenous push over 5 min, followed by 800 mg/m^2 continuous intravenous infusion over 24 h, and LDMTX as 30 mg/m^2 by mouth (PO) every 6 h for six doses. Patients were hydrated for $\geq$ 2 h before HDMTX with intravenous dextrose 5%/0.25 normal saline and 40 meq $NaHCO_3$/liter, until the urine specific gravity was $<$ 1.015 and urine pH was $\geq$ 6.5.

Both treatment groups were given identical leucovorin rescue, 5 mg/m^2 PO every 6 h for five doses, beginning 48 h after the start of MTX. When 44-h MTX plasma concentrations were $>$ 1.0 μM, the dosage of leucovorin was increased according to standardized protocol guidelines and continued until the plasma MTX concentration was $<$ 0.1 μM. Initially, allopurinol (100 mg/m^2 PO three times daily) was used concomitantly with MTX to prevent hyperuricemia. However, allopurinol was not given to subsequent patients in whom MTX's effect on de novo purine synthesis (DNPS) was assessed (i.e., all patients enrolled after 15 February 1994), because of its effects on DNPS (19). Instead of allopurinol, intravenous urate oxidase (uricozyme; Sanofi, Paris, France) was used to prevent hyperuricemia, under the auspices of US FDA IND 43,183. Uricozyme, which has no effect on DNPS, prevents hyperuricemia by enzymatic cleavage of uric acid to more soluble allantoin and has been widely used in Europe for patients with leukemia and lymphoma (20, 21). During the 10-d period after MTX, stomatitis was assessed and graded from 0 to 4 (i.e., least to most severe) according to the National Cancer Institute toxicity criteria (grade 0 = no evidence of stomatitis; grade 1 = painless ulcers, erythema, or mild soreness; grade 2 = painful erythema, edema, or ulcers, but can eat; grade 3 = painful erythema, edema, or ulcers, and cannot eat; grade 4 = requiring parenteral or enteral nutritional support).

Bone marrow and blood sample processing. For all patients, ALL blasts from bone marrow aspirates were obtained at diagnosis (i.e., pretreatment) and 44 h after starting MTX. Samples consisted of 5–10 ml of bone marrow collected in syringes containing 800 U of heparin and were kept on ice until processed ($<$ 1 h). Leukemic blast cells were isolated by Ficoll-Hypaque gradient and washed three times with a solution of Hepes, Hanks' buffered salt solution, and heparin. The final cell yield was determined by hemocytometer and the percent viability by trypan blue exclusion. Cells were aliquoted and assayed for DNPS and MTXPG accumulation, as described below. Blood samples were obtained at times 0, 1, 6, 23, and 44 h from the start of HDMTX; and at times 0, 6, 12, 18, 19, 19.5, 20, 24, and 44 h after the start of LDMTX. Plasma samples were analyzed for MTX by a fluorescence polarization immunoassay (Abbott TDx, Abbott Laboratories, North Chicago, IL). In both treatment groups, samples were obtained before MTX and between 5 and 6 AM on days 1, 2, 3, and 4 (96 h) after starting MTX therapy, for determination of WBC and manual differential. WBC count was determined by Coulter counter (model F+STKR; Coulter Corp., Hialeah, FL), and manual differential was performed using Wright-Giemsa stain with Hematek 2000 (Miles Inc., Kankakee, IL). Peripheral blast count was calculated as WBC $\cdot$ % blasts on peripheral smear ($\times$ 10^9/liter).

DNPS. To determine the rate of DNPS in ALL blasts, radiolabeled purine bases (adenine, guanine) were quantitated after a 2-h ex vivo incubation of lymphoblasts with [^{14}C]formate, according to the previously published method of Matherly et al. (22), and adapted in our laboratory as described (19).

MTX polyglutamylated metabolites. Intracellular MTXPGs were extracted in a buffered solution (Tris, EDTA, and 2-mercaptoethanol) by boiling (100°C for 10 min), then frozen at −80°C until analysis. MTX and six polyglutamylated metabolites ($MTXPG_2$ to $MTXPG_7$) were separated by HPLC, and each metabolite was quantitated by a radioenzymatic assay using a modification of the method reported by Kamen et al. (23), as previously described (16). The column eluent was collected in fractions determined by the elution times of authentic standards for each polyglutamylated metabolite. The limit of detection of this assay was 0.02 pmol/10^6 lymphoblasts; all results were expressed as picomoles of MTX or MTXPG per 10^9 cells.

Pharmacokinetic analysis. Pharmacokinetic parameters were estimated assuming a first-order two-compartment model, using a Bayesian estimation algorithm, as implemented in ADAPT II software (24). The prior distribution of model parameters was based on data from children who received HDMTX or LDMTX, as previously described (25, 26). The area under the concentration versus time curve (AUC) from 0 to 48 h, before the first dose of leucovorin, was calculated by standard trapezoidal methods, and steady state plasma concentration of MTX (Cp_{ss}) was estimated using the fitted 24-h concentration for HDMTX, and as (Cp_{ss} min + Cp_{ss} max)/2 for LDMTX.

Modeling and statistics. The overall objective was to evaluate the effects of dosage (LDMTX or HDMTX), MTX systemic exposure (AUC or Cp_{ss}), and ALL blast MTXPG (total or long-chain) on various endpoints of interest: (*a*) immediate versus delayed onset of cytotoxicity after starting MTX; (*b*) presence or absence of circulating blasts on day 4; and (*c*) dose-limiting toxicity, i.e., grade 0–1 vs. grade 2–4 stomatitis. Logistic regression analysis was used to test the predictive value of the pharmacologic parameters on the outcomes of interest that are binary in nature. Because of the number of subjects in each group, only one factor was analyzed at a time, except for toxicity where the number of subjects permitted regression with two factors at a time. The nonparametric Kolmogorov-Smirnov test was used to compare the distributions of blast MTXPG after LDMTX versus HDMTX, and the log-cell kill in patients treated with HDMTX versus LDMTX or those with blast MTXPG $\geq$ 500 pmol/10^9 cells versus $<$ 500 pmol/10^9 cells. χ^2 or Fisher's exact tests were used to compare the proportions between groups of interest.

For the effect of MTX on DNPS, the percent inhibition of DNPS was calculated as $[(DNPS_{pre} - DNPS_{44H})/DNPS_{pre}] \cdot 100$. The relationship between the percent inhibition of DNPS and the concentration of MTXPG was modeled using the E-max model as previously described (27), where effect (% inhibition of DNPS) = $100 \cdot (1 - e^{-(k \cdot MTXPG)})$, in which k is an estimate of the rate of change between the percent inhibition of DNPS and the concentration of MTXPG. k was estimated by nonlinear weighted least-squares regression. The statistical significance of the model parameter estimate (k) was determined by one degree-of-freedom χ^2 test at the prespecified level of 0.05.

Results

Patients. Between December 1991 and August 1994, 167 patients were randomized to receive either LDMTX or HDMTX, 150 (90%) of whom completed the study and were able

Table I. Demographics of Evaluable Patients Randomized to LDMTX Versus HDMTX

Demographics	LDMTX (n = 76)	HDMTX (n = 74)	P value*
Age (yr)			
< 1	1	1	
1–10	51	55	0.68
> 10	24	18	
WBC ($\times 10^9$/liter)			
< 50	59	60	
50–100	7	8	0.61
> 100	10	6	
Sex			
Male	33	32	0.98
Female	43	42	
DI			
1.16–1.6	19	17	0.77
< 1.16 or > 1.6	57	57	
Ploidy			
> 50 chromosomes	21	17	
⩽ 50 chromosomes	55	56	0.58
Unknown	0	1	
Immunophenotype			
B-lineage	64	65	
T-lineage	12	8	0.47
Unknown	0	1	

*χ^2 or Fisher's exact test.

to be evaluated. Among the 17 patients not evaluated, 5 were considered too unstable to complete the study, 3 refused the additional bone marrow aspirate at 44 h, 1 was diagnosed as having acute myeloid leukemia after being enrolled on the protocol, 1 had an interruption of MTX infusion for a prolonged period of time, and 7 had poor yields of the bone marrow aspirates. There was no difference in the distribution of demographic or biological characteristics between the 76 evaluable patients randomized to LDMTX and the 74 randomized to HDMTX treatment (Table I).

Table II. Intracellular and Extracellular Exposure to MTX after In Vivo Treatment with either LDMTX or HDMTX in 150 Children with Newly Diagnosed ALL

MTX exposure (units)		LDMTX (n = 74)	HDMTX (n = 76)	P value*
Extracellular [plasma]				
Cp_{ss} (μM)	Median:	0.56	12.4	< 0.001
	Range:	0.1–3	5.4–41.2	
$AUC_{0\rightarrow 48h}$ (μM · h)	Median:	21.9	368	< 0.001
	Range:	4.4–86.3	155–1351	
Intracellular [ALL blasts]				
$MTXPG_{2-7}$ (pmol/10^9 blasts)	Median:	443	1290	< 0.001
	Range:	10–3639	144–7519	
$MTXPG_{4-7}$ (pmol/10^9 blasts)	Median:	350	989	< 0.001
	Range:	7–3136	49–7313	

*Determined using Kolmogorov-Smirnov test.

MTX pharmacokinetics. As summarized in Table II, patients who received HDMTX achieved a significantly higher systemic exposure to MTX, defined either by MTX Cp_{ss} or AUC. HDMTX also produced significantly higher intracellular concentrations of total and long-chain MTXPG concentrations in ALL blasts (Table II). Fig. 1 depicts higher MTXPG concentrations in bone marrow blasts 44 h after in vivo exposure to HDMTX compared with LDMTX, extending our earlier findings in a subset of these patients (16). Moreover, MTXPG concentrations were significantly higher in the 129 cases of B-lineage ALL (median $MTXPG_{2-7}$ = 814 pmol/10^9 blasts) compared with the 20 cases of T-lineage ALL (median $MTXPG_{2-7}$ = 252 pmol/10^9 blasts; $P < 0.00001$); the immunophenotype was unknown in one patient. In addition, among the 129 cases with B-lineage ALL, the 33 cases of hyperdiploidy (i.e., > 50 chromosomes) accumulated significantly higher MTXPG (median $MTXPG_{2-7}$ = 1,255 pmol/10^9 blasts) compared with 96 cases with other ploidy (median $MTXPG_{2-7}$ = 632 pmol/10^9 blasts; $P = 0.001$).

Pharmacodynamics of MTX effects on circulating blasts. MTX's effects on peripheral blast count were assessed in the 101 patients (67%) who had a peripheral blood blast count > 0.5 × 10^9/liter before starting MTX. Of the 101 patients meeting this criterion, 95 had peripheral blast counts determined for four consecutive days before the initiation of conventional remission induction chemotherapy. Two endpoints were assessed in these patients: (*a*) immediate versus delayed (i.e., > 24 h) onset of cytotoxicity (n = 101 evaluable patients); and (*b*) presence or absence of circulating blasts on day 4 (n = 95 evaluable patients). Immediate onset of cytotoxicity was defined as a > 10% decrease in circulating blasts within 24 h of starting MTX treatment, and delayed onset was defined as those patients with a < 10% decrease in circulating blasts 24 h after starting MTX. Fig. 2 illustrates four patterns of change in

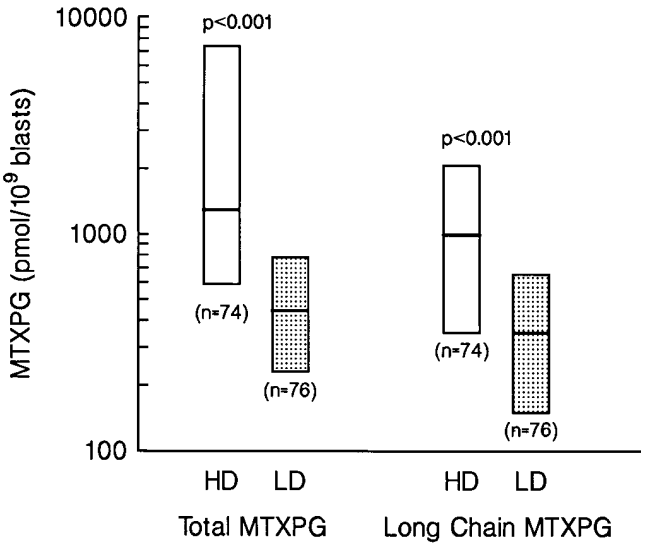

Figure 1. Intracellular concentration of total and long-chain $MTXPG_{4-7}$ in ALL blasts isolated from bone marrow at 44 h after in vivo treatment with either LDMTX or HDMTX in 150 children with newly diagnosed ALL. Data are presented as medians and quartile ranges for HDMTX (*open bars*) and LDMTX (*shaded bars*). P values for LDMTX versus HDMTX were determined by Kolmogorov-Smirnov test.

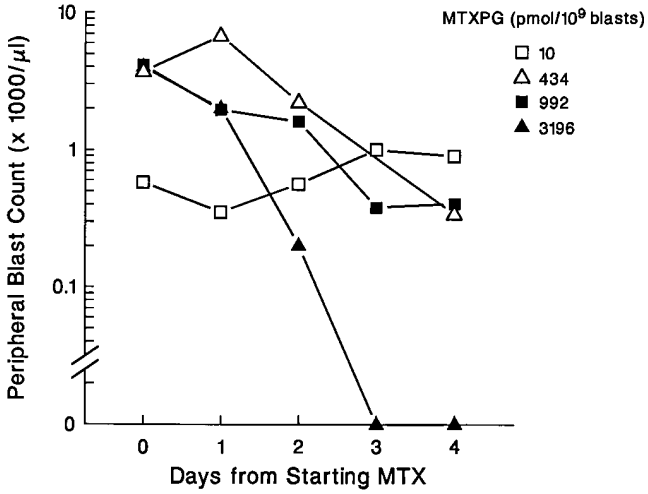

Figure 2. Examples of four patterns of change in circulating blast counts observed after MTX treatment, before starting remission induction chemotherapy. Open symbols depict two patients treated with LDMTX: one who had the lowest MTXPG concentration (□), and one with MTXPG concentrations comparable with the median value for the LDMTX group (△). Closed symbols depict two patients treated with HDMTX: one who had MTXPG concentrations comparable with the median for the HDMTX group (■), and one with MTXPG concentrations greater than twofold higher than the median for the HDMTX group (▲).

circulating blasts observed from pretreatment to day 4: those with no decrease in circulating blasts by day 4 (n = 1), those with a delayed onset of cytoreduction (n = 24 with residual blasts at day 4 and n = 1 with no blasts at day 4), those with an immediate onset of cytoreduction but with residual circulating blasts on day 4 (n = 63), and those with an immediate onset and complete clearing of circulating blasts at day 4 (n = 6). Fig. 3 depicts significantly higher ALL blast concentrations of total

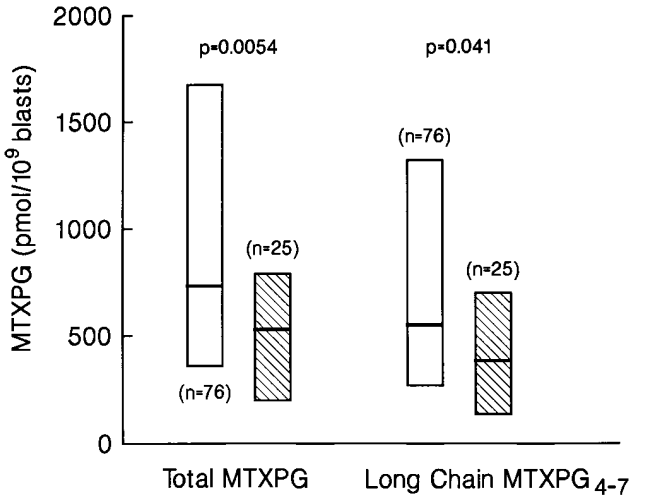

Figure 3. Intracellular concentration of total and long-chain MTXPG in ALL blasts of patients who had an immediate decrease (*open bars*) versus those who had a delayed decrease (*hatched bars*) in circulating blasts after MTX therapy. *P* values are from univariate logistic regression analyses of total and long-chain MTXPG prediction of the onset of response. Medians and quartile ranges depicted.

Table III. Parameters Associated with Onset of Cytotoxicity and Complete Disappearance of Circulating Blasts 96 h after In Vivo Exposure to MTX in Children with Newly Diagnosed ALL

	Antileukemic effects	
MTX exposure	Onset of effect* *P* value§	Clearing of blasts‡ *P* value§
$MTXPG_{4-7}$ (pmol/10^9 blasts)	0.041	0.003
$MTXPG_{2-7}$ (pmol/10^9 blasts)	0.005	0.004
$AUC_{0\rightarrow48h}$ (μM · h)	NS	0.033
Cp_{ss} (μM)	NS	0.034
MTX dose (LDMTX vs. HDMTX)	NS	0.034

*Delayed (i.e., > 24 h) versus immediate decrease in circulating blasts (see Methods); ‡Absence of circulating leukemic blasts by day 4; §Using univariate logistic regression.

and long-chain MTXPG in patients who exhibited an immediate decrease in circulating blasts (n = 76) versus those who had a delayed onset (n = 25). As summarized in Table III, blast concentrations of long-chain and total MTXPG were the only pharmacologic variables significantly associated with the onset of antileukemic effects.

Total and long-chain MTXPG, MTX dose, and MTX systemic exposure were each significant determinants of whether patients were free of circulating blasts at day 4, with blast MTXPG concentration being the most significant variable (Table III). Fig. 4 depicts the concentration of MTXPG measured in ALL blasts at 44 h in patients who had complete clearing of circulating blasts at day 4 (median = 2,793 pmol/10^9 blasts, n = 7) versus those with residual blasts at day 4 (median = 602 pmol/10^9 blasts, n = 88; P = 0.0039 by logistic regression analysis). Likewise, the concentrations of long-chain polyglutamates (i.e., $MTXPG_{4-7}$) were also higher in patients who had complete disappearance of circulating blasts at day 4, as shown in Fig. 4. Of the seven patients who had complete disappearance of circulating blasts, six received HDMTX and only one received LDMTX. There was also a trend toward greater log-cell kill (i.e., log of blast decrease in peripheral blood over 4 d) in patients whose blasts accumulated ⩾ 500 pmol/10^9 blasts (n = 65) compared with patients whose blasts accumulated < 500 pmol/10^9 blasts (n = 30), (mean 3.80 vs. 2.98 log decrease, median 3.5 vs. 2.9 log decrease, P = 0.13), although this did not reach statistical significance given the sample size and heterogeneity of response.

Inhibition of DNPS. The effect of MTX on DNPS in ALL blasts was assessed in 11 patients with nonhyperdiploid B-lineage ALL, the most common biological subtype of ALL. This analysis was restricted to those patients who did not receive concomitant allopurinol, because preliminary studies demonstrated that allopurinol also inhibits DNPS in ALL blasts (19). Among these 11 patients, MTX dose, MTX plasma concentration, and total MTXPG concentration in blasts were not significantly associated with inhibition of DNPS in ALL blasts (P > 0.05). However, the blast concentration of long-chain MTXPG (i.e., $MTXPG_{4-5}$ or $MTXPG_{4-7}$) was a significant determinant of DNPS inhibition (P < 0.001), as depicted in Fig. 5. The concentration of long-chain MTXPG was not significantly different in the 11 patients who were evaluated for DNPS (median

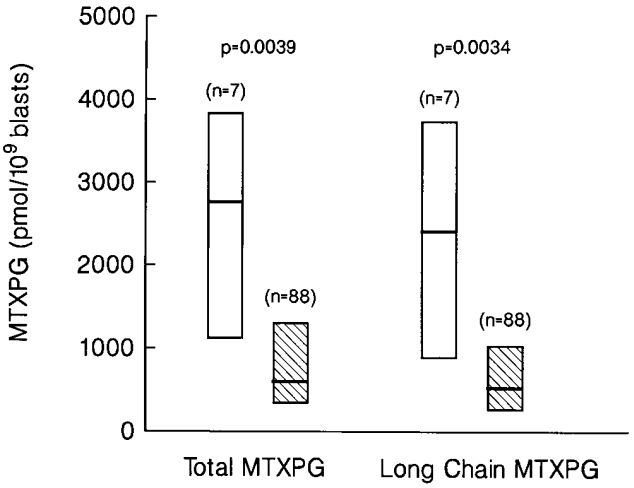

Figure 4. Intracellular concentration of total and long-chain MTXPG in ALL blasts isolated at 44 h from patients who had no residual circulating blasts (*open bars*) versus those who had residual circulating blasts (*hatched bars*) 96 h after in vivo treatment with either LDMTX or HDMTX. *P* values are from univariate logistic regression analyses of total and long-chain MTXPG as determinants of the clearance of circulating blasts. Medians and quartile ranges depicted.

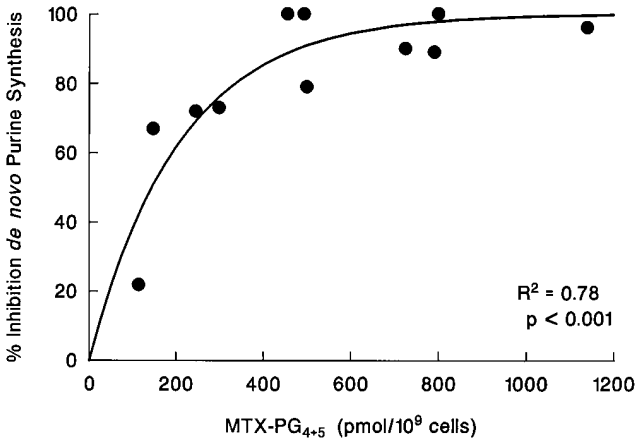

Figure 5. Relation between the percent inhibition of DNPS and the concentration of long-chain MTXPG measured in ALL blasts from bone marrow 44 h after initiating MTX as a single agent in 11 consecutive patients with nonhyperdiploid B-lineage ALL who did not receive allopurinol. Each symbol depicts a patient, and the line depicts the best fit of an E-max pharmacodynamic model to the data. The level of statistical significance for *k* was determined by two tailed Student's *t* test.

$MTXPG_{4-7}$ = 543 pmol/10^9 blasts) and the other 85 patients with nonhyperdiploid B-lineage ALL who were not evaluated for DNPS (median $MTXPG_{4-7}$ = 475 pmol/10^9 blasts; $P = 0.8$). Using an E-max model to characterize the relationship between $MTXPG_{4-5}$ and the inhibition of DNPS, the estimated $IC_{95\%}$ (i.e., the concentration of MTXPG associated with 95% inhibition of DNPS) was 483 pmol/10^9 blasts (95% confidence interval = 370–670 pmol/10^9 blasts). Moreover, long-chain MTXPG concentrations ≥ 483 pmol/10^9 blasts were more likely to be achieved in vivo by administering HDMTX (60/74, 81%) compared with LDMTX (35/76, 46%; $P < 0.0001$).

Pharmacodynamics of dose-limiting toxicity. As summarized in Table IV, the occurrence of grade 2–4 stomatitis was significantly related to several MTX pharmacokinetic parameters, with the best predictor being MTX systemic exposure. The overall incidence of MTX toxicity was relatively low, with only a 15% incidence of ≥ grade 2 stomatitis (22 of 150 patients), and only a 3% incidence of severe stomatitis (five courses associated with grade 3 or 4 stomatitis), all of which occurred after HDMTX. Fig. 6 depicts the proportion of patients who experienced ≥ grade 2 stomatitis at different levels of systemic exposure to MTX (i.e., MTX plasma concentrations). MTX Cp_{ss} within the range of 0.1 to 1.0 μM were observed only in patients who received LDMTX (n = 66; median apparent oral clearance = 412 ml/min/m^2), whereas MTX Cp_{ss} of 10–20 μM were observed only in patients who received HDMTX (n = 48; median systemic clearance = 117 ml/min/m^2). Those with MTX Cp_{ss} in the range of 1.0 to 10 μM were either patients with relatively slow apparent oral clearance after LDMTX (n = 10; median apparent oral clearance = 183 ml/min/m^2) or those with relatively fast systemic clearance after HDMTX (n = 26; median systemic clearance = 173 ml/min/m^2). Patients with $Cp_{ss} > 20$ μM (n = 10) were only those with relatively slow systemic clearance after HDMTX (median systemic clearance = 56 ml/min/m^2). All patients with MTX $Cp_{ss} < 1.0$ μM had MTX plasma concentrations < 1.0 μM at 44 h and therefore received the standard leucovorin rescue (see Methods), whereas 1 of 26 patients with Cp_{ss} between 1.0 and 10 μM, 3 of 48 with Cp_{ss} between 10 and 20 μM, and 4 of 10 with $Cp_{ss} > 20$ μM had 44-h MTX plasma concentrations > 1.0 μM and therefore received escalated leucovorin rescue. Toxicity was not life-threatening in any patient.

Discussion

We demonstrated previously that higher extracellular concentrations of MTX were associated with a lower probability of relapse in children with intermediate-risk ALL treated with antimetabolite-based chemotherapy (25), yet questions were subsequently raised about the rationale for HDMTX in the treatment of this disease (11, 12). To address these questions, we established recently that treatment of ALL patients with HDMTX (i.e., 1,000 mg/m^2) achieves significantly higher con-

Table IV. Parameters Associated with ≥ Grade 2 Stomatitis after In Vivo Exposure to MTX as a Single Agent in 150 Children with Newly Diagnosed ALL

Parameters (units)	*P* value*
Cp_{ss} (μM)	0.0001
$AUC_{0\rightarrow48h}$ (μM · h)	0.0002
MTX dose (LDMTX vs. HDMTX)	0.001
$MTXPG_{2-7}$ (pmol/10^9 blasts)	0.001
$MTXPG_{4-7}$ (pmol/10^9 blasts)	0.003

*Determined by logistic univariate regression analysis. All parameters were positively associated with the occurrence of ≥ grade 2 stomatitis. Analysis adjusting for two independent variables simultaneously was performed to classify the relative importance of each predictive factor as follows: MTX $AUC_{0\rightarrow48h}$ or Cp_{ss} > MTX dose > blast MTXPG concentration (total or long-chain).

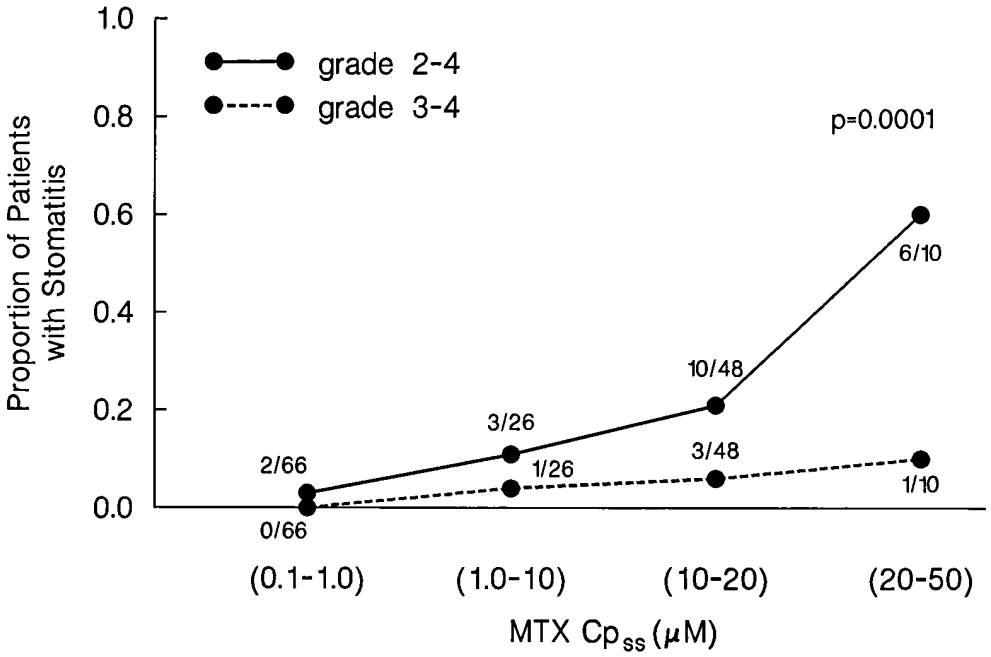

Figure 6. Relation between MTX Cp_{ss} and the proportion of patients who developed stomatitis, i.e., grade 2–4 (*solid line*) and grade 3–4 (*broken line*) up to 10 d after MTX. *P* value is from univariate logistic regression of MTX Cp_{ss} as a determinant of ⩾ grade 2 stomatitis.

centrations of MTX's active polyglutamylated metabolites in ALL blasts, when compared with lower-dose MTX (180 mg/m^2) (16). Because it was not known whether this pharmacologic measurement translated into greater cytotoxicity, the present investigation was undertaken to determine whether higher MTXPG concentrations in the target tissue are associated with greater antileukemic effects in vivo. In 101 evaluable patients who were randomized to receive HDMTX or LD-MTX, MTXPG concentrations in leukemic blasts were found to be significantly higher in patients who had an immediate onset of antileukemic effects, as evidenced by a decrease in circulating ALL blasts within 24 h, and also in those who had complete clearance of blasts within 4 d of single-agent therapy with MTX. Consistent with these findings, inhibition of DNPS in ALL blasts was significantly related to blast accumulation of MTXPG after in vivo treatment with MTX, and the concentration of long-chain MTXPG required to produce 95% inhibition of DNPS (i.e., 483 pmol/10^9 blasts) was achieved in a significantly higher proportion of patients treated with HDMTX compared with LDMTX (81 vs. 46%). Collectively, these data indicate that the concentration of MTXPG in ALL blasts is an important determinant of antileukemic effects in vivo and that HDMTX is more likely than LDMTX to achieve cytotoxic concentrations in the target tissue.

It is recognized that the ultimate measure of antileukemic effects is long-term disease-free survival, rather than initial cytotoxicity. However, to define the intracellular disposition and cytotoxic effects of individual agents for childhood ALL, it is necessary to undertake such studies within the first few days after diagnosis (i.e., "up-front window studies"), since > 95% of patients will achieve complete remission after 4–6 wk of multiagent remission induction therapy. Once multiagent therapy has been initiated, it is not possible to assess the cytotoxic effects of single chemotherapeutic agents, and after complete remission has been achieved, there are no leukemic blasts available for pharmacologic studies. Moreover, studies from several institutions have shown that the initial leukemic response, assessed by changes in circulating or bone marrow blasts during the initial weeks of treatment, is a significant independent prognostic factor related to long-term disease-free survival in childhood ALL (28–30). In this regard, recent data from our institution indicate that the absence of circulating blasts by day 8 of remission induction therapy is associated with significantly better long-term disease-free survival (31). Therefore, by establishing that higher ALL blast concentrations of MTXPG are significantly associated with an immediate onset and complete clearance of peripheral blasts, the present study provides evidence that HDMTX is superior to LDMTX in the treatment of childhood ALL.

The biochemical and molecular mechanisms by which MTX exerts its principal cytotoxic effects in leukemic blasts are complex and not fully elucidated (5, 32, 33). MTX and its active MTXPG metabolites are known to inhibit dihydrofolate reductase, thereby lowering cellular pools of reduced folates that are necessary cofactors for the synthesis of purines and pyrimidines. The polyglutamylated metabolites of MTX are also more potent than MTX at inhibiting thymidylate synthase and enzymes catalyzing DNPS, and these metabolites are retained longer inside cells (32, 34, 35). In vitro experiments have shown that long-chain polyglutamates of MTX (i.e., $MTXPG_{4-5}$) are more potent inhibitors of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, compared with MTX or short-chain MTXPG (i.e., with one or two glutamyl residues) (34). Inhibition of DNPS is also considered a primary mechanism for synergism between MTX and mercaptopurine (36). Thus, there are multiple mechanisms whereby greater intracellular accumulation of MTXPG may enhance the antileukemic effects of MTX (32, 35). In the present study, lymphoblast concentration of long-chain MTXPG was the only variable significantly associated with the extent of inhibition of DNPS in ALL blasts. To our knowledge, this represents the first human study to demonstrate the importance of MTXPG accumulation in ALL blasts as a determinant of methotrexate's antileukemic effects, in vivo.

Whitehead et al. (37) previously reported an improved event-free survival in children whose ALL blasts accumulated total MTXPG > 500 pmol/10^9 blasts after ex vivo incubation of blasts with ^{3}H-MTX. In their subsequent in vitro study, blasts from patients with hyperdiploid ALL were found to accumulate more MTXPG than those with nonhyperdiploid ALL blasts, a finding that could explain the better prognosis observed in patients with hyperdiploid B-lineage ALL treated with antimetabolite-based chemotherapy (10, 38). Patients with T-lineage ALL generally have a worse prognosis than patients with B-lineage ALL, especially when treated with conventional antimetabolite chemotherapy. In this regard, Bertino et al. (39) found that B-lineage lymphoblasts accumulated higher concentrations of long-chain MTXPG than T-lineage blasts, after in vitro incubation with MTX. We have extended these findings in our in vivo studies, establishing significant ploidy and lineage differences in accumulation of MTXPGs in ALL blasts (16). The higher concentrations of MTXPGs found in cases of B-lineage ALL appear to be related in part to higher constitutive and inducible folylpolyglutamate synthetase activity in B-lineage compared with T-lineage lymphoblasts (40), although differences in membrane transport may also be a contributing factor (16). Among the 20 T-lineage cases enrolled on the present study, HDMTX achieved significantly higher blast concentrations of $MTXPG_{4-6}$ (334 pmol/10^9 blasts) compared with LDMTX (145 pmol/10^9 blasts), and only 3 of 8 patients (37%) treated with HDMTX and none of 12 patients (0%) treated with LDMTX had blast concentrations of long-chain polyglutamates > 483 pmol/10^9 blasts at 44 h. Of the 13 patients with T-lineage ALL evaluable for peripheral blast response in this study, none had complete disappearance of circulating blasts by day 4 after therapy with either LD-MTX (n = 5) or HDMTX (n = 8). It is possible that the MTXPG concentration required to elicit antileukemic effects may be different (i.e., higher) in T-lineage ALL blasts compared with B-lineage ALL blasts. Although improved results in T-lineage ALL have been credited to the use of HDMTX (3–5 grams/m^2) in two recently reported clinical trials (9, 41), it remains to be determined whether MTX doses higher than those used in the present study (i.e., > 1,000 mg/m^2) would achieve higher MTXPG concentrations and hence greater antileukemic effects in patients with T-lineage ALL.

This study provides evidence that higher concentrations of MTX's active metabolites in the target tissue are associated with greater antileukemic effects in B-lineage ALL, and that HDMTX (1,000 mg/m^2 intravenously over 24 h) is superior to LDMTX in achieving these intracellular concentrations. Moreover, the difference between HDMTX and LDMTX was evident even though the dose of LDMTX in this study was higher than conventional LDMTX in most treatment protocols (i.e., 180 vs. 20–40 mg/m^2). HDMTX also offers the potential advantage of eradicating extramedullary leukemia by producing cytotoxic concentrations in "sanctuary" sites where MTX does not readily distribute (e.g., testes, cerebrospinal fluid) (10). Collectively, these data provide a strong rationale for HDMTX in the treatment of childhood ALL. Future studies should now focus on defining the optimal strategy for using HDMTX to treat this disease, addressing issues such as the proper dosage (e.g., 1,000 vs. 5,000 mg/m^2), the most appropriate duration of infusion (e.g., 2 vs. 24 h), and the most effective antileukemic agents to combine with HDMTX.

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