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Accumulation of Methotrexate and Methotrexate Polyglutamates in Lymphoblasts at Diagnosis of Childhood Acute Lymphoblastic Leukemia: A Pilot Prognostic Factor Analysis

By V. Michael Whitehead, David S. Rosenblatt, Mary-Jane Vuchich, Jonathan J. Shuster, Anne Witte, and Denise Beaulieu

Lymphoblasts in bone marrow samples, obtained from 43 children with acute lymphoblastic leukemia at diagnosis, were incubated with 1.0 μmol/L [^{3}H] methotrexate for 24 hours in vitro. Nonexchangeable methotrexate and methotrexate polyglutamates were separated and quantitated. Event-free survival at 5 years was 38% ± 9% for all 43 patients (27 failures), and 44% ± 10% for the 35 with non–T, non–B-cell acute lymphoblastic leukemia (20 failures). Of these 35 children, those whose lymphoblasts accumulated more than 100 pmol methotrexate and 500 pmol methotrexate polyglutamates per billion cells experienced better 5-year event-free survival than those whose lymphoblasts did not (65% ± 12% v 22% ± 9%, $P = .010$). This difference characterized "good-risk" patients who were female ($P = .014$), less than age 7 at diagnosis ($P = .005$), or had low initial white blood cell counts (less than 20×10^9/L, $P = .018$). Findings were similar for the 43 children with acute lymphoblastic leukemia and for the "good-risk" children in this total group. Thus, the ability of lymphoblasts to accumulate methotrexate and form methotrexate polyglutamates may be important to the curative properties of current therapy of acute lymphoblastic leukemia in children, particularly for "good-risk" patients. In such patients, inherent rather than acquired drug resistance may be the initial event leading to treatment failure.
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MANY CHILDREN who are treated for acute lymphoblastic leukemia (ALL) today achieve long-term drug-free disease control or cure.[1] The availability of a number of active anti-leukemic drugs combined with knowledge of how to combine and schedule their use to best effect, knowledge gained from numerous clinical trials, has led to successive increases in event-free survival (EFS) of these children.[2-4]

Unfortunately, many children still experience recurrence of their leukemia. With relapse, the lymphoblasts that reappear display resistance to drugs which worked before.[5] It is not known whether such resistance is present initially at diagnosis or develops later during therapy presumably through mutation. It is also not known to which of the several established anti-leukemic drugs the lymphoblast first displays resistance. We thought there was a good chance that methotrexate (4-NH_2-10-CH_3PteGlu, MTX) might be that drug.

MTX is an analog and antagonist of the vitamin folic acid and an important anti-cancer drug. After 40 years[6] of experience in its use in many different cancers, its effectiveness is universally recognized. In the current treatment of childhood ALL, MTX is administered intrathecally to prevent central nervous system (CNS) leukemia[7]; intravenously in intermediate or high doses, followed by leucovorin rescue, as intensification therapy[8,9]; and once or twice weekly by mouth during continuation therapy.[10]

Various mechanisms have been described by which cultured mouse and human leukemic cells become resistant to MTX. These include decreased MTX transport,[11] gene amplification resulting in high levels of dihydrofolate reductase (DHFR),[12] an altered affinity of DHFR for MTX,[13,14] and decreased accumulation of MTX polyglutamates (MTXPG).[15,16]

MTX is a substrate for the enzyme folate polyglutamate synthetase (FPGS),[17] which converts it to MTXPG having up to six additional γ-linked glutamyl residues. Cells can accumulate and retain high levels of MTXPG, resulting in prolonged inhibition of DNA synthesis.[18-21] MTXPG, like MTX, have a high affinity for DHFR.[21-24] Moreover, and unlike MTX, they inhibit other folate enzymes, including thymidylate synthase[25,26] and the transformylases used in purine synthesis.[27] These properties suggest strongly that MTXPG, particularly long-chain forms containing four or more total glutamates, play a critical role in the cytotoxic action of MTX.[28]

In a study reported earlier,[29] lymphoblasts were obtained at diagnosis from children with ALL, were incubated with MTX in vitro and nonexchangeable MTX and MTXPG were quantitated using high performance liquid chromatography (HPLC). These lymphoblasts accumulated predominantly long-chain MTXPG, principally MTX pentaglutamate. While there was little patient-to-patient variation in MTXPG distribution by glutamyl chain-length, there was great variation in the levels of MTX and MTXPG.[29]

The present pilot study was undertaken to measure the extent of MTX metabolism in vitro in lymphoblasts obtained from children with ALL at the time of diagnosis. Findings were compared with known prognostic features and response

From the Penny Cole Hematology Research Laboratory, McGill University-Montreal Children's Hospital Research Institute, Canada; and the Department of Statistics, University of Florida, Gainesville.

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Address reprint requests to V. Michael Whitehead, MD, Hematology Service, Montreal Children's Hospital, 2300 Tupper St, Montreal, Quebec, H3H 1P3 Canada.

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to treatment to generate testable hypotheses for larger studies. Results showed that patients whose lymphoblasts accumulated high levels of both MTX and MTXPG survived better than those whose lymphoblasts did not. This was particularly evident in so-called "good-risk" children with non–T, non–B-cell ALL. Preliminary results have been reported.[30]

MATERIALS AND METHODS

Patients studied. ALL was diagnosed in 79 children at the Montreal Children's Hospital (MCH) between January 1979 and May 1985. With informed consent, an additional 1 to 2 mL bone marrow sample was obtained from 46 of these patients during the initial bone marrow aspiration. Normal marrow elements comprised 51% of the cells in one sample. In two other samples, results were not obtained because of technical difficulties. Of the 43 samples giving satisfactory results, 38 contained 90% or more lymphoblasts. In the other five patients, lymphoblasts ranged from 70% to 88% of bone marrow cells. These patients were included since their category of extent of MTX metabolism, presuming 100% lymphoblasts, did not change.

There were 20 females and 23 males. Ages at diagnosis ranged from 19 to 180 months. The white blood cell (WBC) count at diagnosis ranged from 1.6 to 656.0 × 10^9/L. Thirty-five patients had non–T, non–B-cell ALL. Of these, 17 had early pre–B and five pre–B-cell ALL. In 13 patients, the cytoplasmic Ig status was not known. Five patients had T-cell, and single patients each had combined pre-B and T-cell, B-cell, and uncharacterized ALL. Two patients had a Philadelphia chromosome. One of them was thought to have chronic myelocytic leukemia (CML) in blast crisis.

Among the 35 children with non–T, non–B-cell ALL, those who were female, young, or whose initial WBC count was low were considered to be "good-risk" (Table 1). Young was arbitrarily defined as less than age 7 and a low WBC count as less than 20 × 10^9/L.

Treatment of the leukemia. All patients were treated at the MCH. They received a variety of treatments. Of the 35 children with non–T, non–B-cell ALL, 26 were treated on Pediatric Oncology Group (POG) 8036,[31] 4 on POG 7623,[32] and 2 on Cancer and Leukemia Group B (CALGB) 7611.[8] Three received other treatments. Four patients with T-cell ALL were treated on POG 7837[33]; two received other treatments. The patients with B-cell and uncharacterized ALL were treated on POG 8106[34] and CALGB 7611,[8] respectively. MTX was an important component of all these treatments.

Incubation of lymphoblasts with [^{3}H]MTX. All studies of MTX metabolism were performed on fresh lymphoblasts. Delay of several hours resulted in a decrease in such metabolism. Cells were sedimented for 1 hour at unit gravity and room temperature in 6% dextran in saline. The buffy coat was washed twice with sterile Hanks' balanced salt solution lacking phenol red and $NaHCO_3$, pH 7.4 (Flow Laboratory, Toronto, Ontario, Canada).

Five million nucleated cells were incubated in 2.0 mL modified Eagle's minimal essential medium (MEM, Flow Laboratory) to which was added 26 mmol/L $NaHCO_3$, 1.25 mmol/L pyruvate, 8.3 mmol/L dextrose, and 0.025 mmol/L ferric nitrate, and containing 10% fetal calf serum (GIBCO Co, Burlington, Ontario, Canada) and 1.0 μmol/L 3',5'-7-^{3}H-MTX (Amersham Co, Oakville, Ontario, Canada; Moravek Co, Brea, CA) in a P-35 culture dish (Falcon Co, Pointe Claire, Quebec, Canada) for 24 hours at 37°C in 5% CO_2-95% O_2. This exposure of lymphoblasts to MTX was chosen to approximate in vivo conditions during administration of intermediate-dose MTX to children with ALL.[8]

After incubation, cells were washed twice and then incubated in Hanks' for 1 hour to allow efflux of exchangeable MTX and MTXPG. After a further wash, cells were lysed by combined sonication (Sonic Dismembrator, Fisher Scientific Co, Montreal, Quebec, Canada) and freezing. Extracts were prepared for gel filtration by boiling for 10 minutes. For HPLC, trichloracetic acid was added to a concentration of 10% to extracts.

The level of total MTX and MTXPG together was measured in sample supernatants (RackBeta liquid scintillation counter; LKB-Wallac, Montreal, Canada). For the initial 30 patients, MTX and MTXPG were separated and quantitated using Sephadex G-15 (Pharmacia Fine Chemicals, Montreal, Quebec, Canada) gel filtration chromatography.[22] HPLC (Waters Associates, Milford, MA)[29,35] was used for the remaining 13 patients. The ability of these two methods to separate MTX from total MTXPG was compared in nine patients. Analysis of results using linear regression yielded a standard error of prediction of 8.1% and an R^2 = .86, indicating that gel chromatography is estimated to explain 86% of the variation of HPLC.[36] This represents a very high correlation of these two methods. Because gel filtration did not separate MTXPG of different chain lengths one from another, results for all patients are expressed as total MTX and total combined MTXPG.

Statistical analysis. The logrank test[37] was used to compare EFS curves, which were constructed by the method of Kaplan-Meier,[38] using standard errors of Peto et al.[39]

RESULTS

Accumulation and polyglutamylation of MTX in lymphoblasts. All bone marrow samples took up MTX and formed MTXPG. There was wide variation in the level of unmetabolized MTX, from 10 to 1,767 pmol/10^9 cells, a 177-fold difference. Levels of MTXPG also ranged widely, from 93 to 3,285 pmol/10^9 cells, a 35-fold difference.

Table 1. Relation of EFS to Levels of MTX and MTXPG in "Good-" and "Poor-Risk" Subgroups of Patients With Non–T, Non–B-Cell ALL

Risk	MTX <100 or MTXPG <500 (pmol/10^9 cells)			MTX >100 and MTXPG >500 (pmol/10^9 cells)			
Subgroups*	N	Fail	Expected	N	Fail	Expected	P Value†
Female	9	6	3.0	7	0	3.0	.014
Male	10	9	6.5	9	5	7.5	.19
Age <84 mos	16	13	7.5	11	2	7.5	.005
Age >84 mos	3	2	1.8	5	3	3.2	
WBC <20	10	8	4.2	12	3	6.8	.018
WBC >20	9	7	6.4	4	2	2.6	.66
Total	19	15	9.6	16	5	10.4	.016

*"Good-risk," female, age <84 mos or WBC <20 × 10^9/L. "Poor-risk," male, age >84 mos or WBC >20 × 10^9/L.

†Two-sided logrank test.

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Levels of greater than 100 pmol MTX and of greater than 500 pmol MTXPG per 10^9 lymphoblasts were arbitrarily defined as high. The MTX level was high in 28 patients, of whom 23 had non–T, non–B-cell ALL. The level of MTXPG was high in 22 patients and 21 had non–T, non–B-cell ALL. Levels of both MTX and MTXPG were high in 17 patients, and 16 had non–T, non–B-cell ALL (Table 1).

Outcome of therapy. One patient believed to have had CML in blast crisis did not achieve remission. The levels of MTX and MTXPG in his lymphoblasts were both high. He did not receive MTX. Forty-two patients or 98% achieved complete clinical remission (CCR). Two patients died of infection 4 and 20 months after achieving CCR. Both patients had high MTX levels and low MTXPG levels in their lymphoblasts. Twenty-four patients experienced relapse of their leukemia 2 to 87 months after attaining CCR. Sites of relapse were the bone marrow in 18, bone marrow and CNS in 3, bone marrow and testes in 2, and testes in 1. All patients in CCR have been followed 4 years and more. Overall 5-year EFS was 38% ± 9% among the 43 patients (27 failures) and 44% ± 10% among the 35 patients with non–T, non–B-cell ALL (20 failures). Overall 5-year survival for all 79 patients diagnosed during the study interval was 41% ± 6% (47 failures).

Relation of individual MTX and MTXPG levels in lymphoblasts to EFS. For the 35 patients with non–T, non–B-cell ALL, 5-year EFS was better for those whose lymphoblast MTX level was high compared with low, 57% ± 12% versus 25% ± 15% ($P = .024$). For these 35 patients, the EFS at 5 years was better for children whose lymphoblasts accumulated high versus low levels of MTXPG, 57% ± 14% versus 29% ± 12%, $P = .14$, respectively. This difference was not significant.

In the "good-risk" subgroups of the 35 patients with non–T, non–B-cell ALL, there was a significant correlation of EFS with level of lymphoblast MTX in females, $P = .014$ and young children, $P = .009$. No significant correlations were found in males, older children, and in those with low or high initial WBC counts. There was a suggestive correlation of lymphoblast MTXPG level and EFS in those with low initial WBC counts, $P = .062$, and in young children, $P = .052$. There was no significant correlation between lymphoblast MTXPG levels and EFS in males or females, in older children, and in those with high initial WBC counts.

Similar correlations were found when either MTX or MTXPG levels were related to EFS in the 43 patients and in the "good-risk" patients among them.

Relation of combined MTX and MTXPG levels in lymphoblasts to EFS. Children whose lymphoblasts accumulated both high MTX and high MTXPG levels together experienced significantly better EFS than those whose lymphoblasts accumulated either low MTX or low MTXPG or both. For the 43 patients, EFS at 5 years was 65% ± 15% versus 22% ± 9%, $P = .010$. For the 35 patients with non–T, non–B-cell ALL, EFS at 5 years was 69% ± 15% versus 27% ± 11%, $P = .016$ (Fig 1). There was a significant correlation between combined MTX and MTXPG levels and EFS in "good" but not in "poor-risk" subgroups based on sex, age, and initial WBC count in the 35 patients with non–T, non–B-cell ALL (Table 1). Similar significant correlations between high and low combined MTX and MTXPG levels and EFS were found in "good" but not "poor-risk" subgroups in all 43 patients.

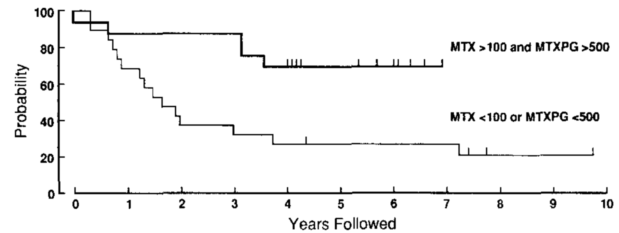

Fig 1. Relation of combined MTX and MTXPG levels in lymphoblasts to EFS in 35 children with non–T, non–B-cell ALL. There were 16 children with lymphoblast MTX greater than 100 and MTXPG greater than 500 pmol/10^9 cells (five failures), and 19 children with MTX less than 100 or MTXPG less than 500 pmol/10^9 cells (15 failures).

DISCUSSION

The EFS of the patients studied here was disappointing and less than the 50% or greater reported in recent studies,[1-4,10] despite the fact that 36 of them were registered on and are part of such studies.[8,31-34] There was no excess of poor-risk immunophenotypes (T–, B–, or pre–B-cell ALL), nor of poor-risk patients treated on POG 8036 (11 of 26, or 46%, poor risk).[31] The EFS for all 79 patients was similar to that of those studied, excluding the possibility of having selected poor-risk patients perhaps based on increased marrow cellularity.

Thirty-five children (81%) had non–T, non–B-cell ALL, so-called common ALL. Of the 8 children with other immunophenotypes (T-cell, B-cell, and unknown), 7 had low levels of MTX and/or MTXPG, and 7 (6 with low levels) experienced relapse. This negative association probably explains why their inclusion with the non–T, non–B-cell ALL patients reduced overall EFS, but not the close relationship to MTX metabolism in lymphoblasts. Indeed, qualitative conclusions in relation to MTX metabolism and EFS in patients with non–T, non–B-cell ALL and in the "good-risk" subgroups of them (Fig 1, Table 1) were true for all 43 patients as well. There were too few children with T- and B-cell ALL to establish or exclude a relationship between EFS and lymphoblast metabolism of MTX in vitro in them. However, such a relationship might have been demonstrated if a larger number of patients had been studied.

Thus, the positive correlation of EFS and combined high intracellular MTX and MTXPG levels in the study is a feature of the children with non–T, non–B-cell ALL. For these 35 patients, there was considerable uniformity of treatment. Two thirds were treated on regimens 1 and 2 of POG 8036, which included considerable exposure to MTX during consolidation.[31] Regimen no. 2 consisted of regimen no. 1 with the addition of high-dose MTX infusions during maintenance therapy. Many of the 12 other patients with non–T, non–B-cell ALL received the same drugs used in these regimens, but in different doses and schedules.[8,32] Nevertheless, differences in treatment may well have influenced outcome in individual patients.

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The increased EFS in patients whose lymphoblasts accumulated either high MTX or high MTXPG levels suggested a relation between MTX metabolism by lymphoblasts and response to therapy. However, the correlations were weak. Indeed, the correlation of EFS appeared better with MTX levels than with MTXPG levels, achieving statistical significance in the 35 children with non–T, non–B-cell ALL ($P = .024$). This is contrary to current views regarding the importance of MTXPG to MTX cytotoxicity.[28] Additional significant correlations were observed in certain "good-risk" subgroups, further suggesting a relation between individual MTX and MTXPG levels in lymphoblasts and overall effectiveness of treatment.

Strong correlation between MTX metabolism in lymphoblasts in vitro and EFS was obtained when both MTX and MTXPG levels were analyzed together. Children whose lymphoblasts accumulated high levels of both MTX and MTXPG experienced significantly better EFS than those whose lymphoblasts accumulated high levels of only one or the other, or neither (Fig 1).

A feature of non–T, non–B-cell ALL as currently treated is the ability to define groups of patients more or less likely to respond well to treatment, so-called "good-" and "poor-risk" subgroups, based on a variety of prognostic features. Sex and age are the most important host factors. Females do better than males, even allowing for testicular relapse.[3,40,41] Excluding babies, younger children do better, with the choice of age to divide younger from older children varying from study to study. The total initial WBC count is the most important disease feature: the lower it is the better the EFS.[3,40,41]

When correlations were sought by these risk factors, the extent of lymphoblast metabolism of MTX in vitro was found to be closely linked to EFS in the "good-risk" subgroups in patients with non–T, non–B-cell ALL (Table 1). No such correlations were found in "poor-risk" patients. Indeed, most of these experienced relapse, including those with high lymphoblast MTX and MTXPG levels. Thus, the close association between the extent of MTX metabolism by lymphoblasts and the success of polychemotherapy appears to be restricted to "good-risk" children with non–T, non–B-cell ALL. The small number of patients in this study and incomplete cytogenetic data in them precluded further analysis of patient sub-groups and correlation with other prognostic factors (eg, DNA index and chromosomal translocations).

The finding that lymphoblast MTX and MTXPG levels were independently related to EFS to some degree suggests that these two measures reflect different components of MTX metabolism by lymphoblasts. The nature of these two components is not known at this time. Accumulation of high levels of intracellular MTXPG presumably requires both excess substrate MTX and sufficient FPGS activity. Exposure of lymphoblasts to 1.0 μmol/L [^{3}H]MTX in vitro would be expected to provide sufficient intracellular substrate MTX. Therefore, differences in levels of lymphoblast MTXPG may reflect differences in FPGS activity.

Differences in activity of gammaglutamyl hydrolases (GGH), which hydrolyze MTXPG to MTX, may affect this relationship as well. Using an inhibitor of GGH, 2-mercaptomethylglutaric acid (MMGA), inhibition of GGH hydrolysis of MTXPG has been demonstrated in fresh human leukemic cells, both lymphoblasts and myeloblasts.[42] Thus, the levels of MTXPG formed in lymphoblasts in vitro probably reflect both synthesis and breakdown of MTXPG by FPGS and GGH, respectively.

It is intriguing to speculate that the level of nonexchangeable MTX may relate to drug transport by lymphoblasts. This requires further study. Though numbers were small, there was no difference in EFS of patients with isolated low MTX or low MTXPG compared to those with both low MTX and MTXPG.

There is considerable evidence that treatment failure in childhood ALL is associated with the appearance of lymphoblasts that have undergone mutation and become drug-resistant.[1,43] Attempts to determine whether MTX metabolism in lymphoblasts is altered at relapse compared with diagnosis have failed to date because of difficulties in obtaining pure enough preparations of lymphoblasts at relapse. The present findings linking the extent of lymphoblast metabolism of MTX at diagnosis with EFS are not in disagreement with lymphoblasts eventually undergoing mutation, but would favor inherent rather than acquired drug resistance as the initial cause of treatment failure in some children with ALL.

A number of classifications of childhood ALL exist, including those based on lymphoblast morphology, immunophenotype, and DNA content.[4] The differences in levels of lymphoblast MTX and MTXPG from patient to patient[29] suggested that a pharmacogenetic classification of childhood ALL might be possible as well. The findings reported in this pilot study suggest that such a classification may have clinical relevance.

This hypothesis will be tested prospectively in a large number of patients in the next frontline ALL study of the POG, ALinC 15. Preliminary results indicate (POG 8901) that the study of MTX metabolism in lymphoblasts can be performed successfully in many POG institutions.

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