Clinical Correlates of in Vitro Effect of Methotrexate on Acute Leukemia Blasts

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SUMMARY

The effect of $1 \times 10^{-6}$ M methotrexate (MTX) on tritiated thymidine-incorporation was studied after 4 hr treatment of suspensions of human acute leukemia blasts in vitro. In previously untreated cases, on the average, MTX suppressed tritiated thymidine incorporation by blood blasts but stimulated incorporation by marrow blasts. The lower the blast count in the patient's blood the greater was the in vitro suppression. The lymphoblasts of children were more sensitive to MTX than were the lymphoblasts of adults. When patients with acute lymphoblastic leukemia were put into remission and maintained with MTX, there was a correlation between sensitivity to MTX in vitro and length of remission. There was also a correlation between in vitro effect of MTX and duration of survival, and the correlation was better than that between blast count and survival.

Surprisingly, in patients with nonlymphoblastic leukemia there was also a good correlation between initial sensitivity to MTX in vitro and duration of survival despite the fact that most of these patients were never treated with MTX. In this group, the blast count did not correlate with survival.

The suppression of tritiated thymidine incorporation could not be prevented by simultaneous addition of purines or reversed by delayed addition of folic acid, suggesting that the suppressive effect was due to rapid in vitro kill of leukemic blasts.

INTRODUCTION

MTX$^3$ is an antifolate that can cure humans of malignant disease (4), but unfortunately its antitumor spectrum is narrow. The basis for its greater cytotoxic effect against one type of human neoplastic cell as opposed to another, or for neoplastic cells as opposed to normal proliferating cells, is unknown although much is known about its biochemical pharmacology from model systems. The current study seeks to identify differences in effects of MTX on blasts from patients with acute leukemia and to relate these differences to various clinical aspects of the disease. The approach used was one suggested by studies in a model system.

When murine L5178Y lymphoblasts in permanent culture are treated with MTX, incorporation of TdR-$^3$H may be acutely suppressed. The greater the suppression the more sensitive are the cells to the cytotoxic effects of MTX as measured by cloning; in fact, the effect on TdR-$^3$H incorporation can be used as a biochemical substitute for cloning (7).

Human acute leukemic cells might be similar to the murine lymphoblasts. In vitro treatment with MTX might suppress TdR-$^3$H incorporation in those cells most sensitive to the cytotoxic effects of the antifolate. If so, the in vitro effects of MTX on TdR-$^3$H incorporation might predict the responsiveness to MTX therapy.

MATERIALS AND METHODS

MTX (sodium salt) and folic acid (Leucovorin) for the in vitro studies were gifts from Cyanamid (Lederle) of Canada, Montreal, Canada; Fischer's medium and horse serum were obtained from Grand Island Biological Company, Grand Island, N.Y. The horse serum was pooled from 5 separate batches, but it was not decomplemented. TdR-$^3$H (specific activity, 1.9 mCi/mole) was from New England Nuclear, Boston, Mass.; heparin (with benzyl alcohol preservative) was from Abbott Laboratories, Montreal, Canada; purines (hypoxanthine, inosine, adenosine, guanosine, deoxyadenosine) were from Nutritional Biochemical Corporation, Cleveland, Ohio.

Leukocyte-rich plasma was decanted from heparinized venous blood (10 units/ml) after the erythrocytes had settled by gravity. The leukocytes were separated from the plasma by centrifugation (900 $X$ $g$, 4$^\circ$, 4 min) and resuspended to 1 to 2 $X$ 10$^6$/ml in EHS. The suspensions of peripheral blood leukocytes contained between 3 and 92% leukemic blasts. Suspensions of marrow blasts were prepared by aspirating 1 ml of marrow from the posterior iliac spine into a syringe containing 100 units of heparin, mixing the marrow with 10 ml of ice-cold EHS, dispersing the cells from the marrow particles gently with a Pasteur pipet, centrifuging the cells (900 $X$ $g$, 4$^\circ$, 5 min), and resuspending them to 1 to 2 $X$ 10$^6$/ml in EHS. In marrow studies the leukemic blasts comprised more than 90% of the DNA-synthesizing cells.

One-ml aliquots of the cell suspensions were delivered into 5-ml plastic tubes (Falcon Plastics, Oxnard, Calif.) with 0.1 ml EHS or MTX to make the final desired MTX concentration

1 This work was supported by National Cancer Institute of Canada Grant 383-3119-01 the Medical Research Council of Canada Grant 321-3119-08.

2 Scholar of the Leukemia Society of America.

3 The abbreviations used are: MTX, methotrexate; TdR-$^3$H, tritiated thymidine; EHS, Eagle's medium supplemented with 10% pooled horse serum; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; AUL, acute undifferentiated leukemia; non-ALL, non-lymphoblastic leukemia; DHFR, dihydrofolate reductase.

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and were incubated at 37° in an atmosphere of 5% CO₂. At intervals during the next 24 hr the tubes were transferred from the CO₂ incubator to a Dubnoff shaking water bath at 37°, reequilibrated with 5% CO₂ by breathing several times into each tube, and stoppered. The cells were pulsed with TdR-3H and incorporation of TdR-3H into DNA was measured as a rate over a 28-min period by previously published methods using liquid scintillation techniques (10). Control rates were expressed as fmoles of thymidine incorporated per min of incubation per million blasts. When the standard deviation of the rate was greater than 20% of the value of the rate, the data were discarded. This rule was followed rigidly so that the data could be plotted in the logarithmic form and comparisons could be made between differences which, while they were large in relative terms, were small on a linear scale. Rate constants measured in the presence of MTX were then expressed as a percentage of the rate constant in the absence of MTX. Thus, a rate of 100% in the presence of MTX would indicate that MTX had neither suppressed nor simulated incorporation.

A total of 56 cases of previously untreated acute leukemia have been studied to date. The diagnosis of leukemia and definition of type of disease were according to accepted morphological criteria as ALL or AML. Morphological variants such as acute myelomonocytic leukemia were included as AML. When the cells were not clearly identifiable as ALL or AML, the disease was designated as AUL and classed with the nonlymphoblastic group. There were 25 cases of ALL (17 children), 24 cases of AML (7 children), and 7 cases of AUL (6 children). Patients over 15 years of age at diagnosis were classified as adults.

In 23 of the patients with ALL, induction of remission was successful with vincristine and prednisone. These patients were then maintained with MTX. In the majority (19 cases) the maintenance was Regimen D, consisting of MTX up to 15 mg/sq m/day for 5 days approximately every 2 weeks with every 3rd course of MTX followed by reinduction with vincristine-prednisone for 2 weeks. Two cases were maintained with MTX, 15 mg/sq m biweekly, and 2 cases were maintained with a combination of methotrexate and cytosine arabinoside. Patients relapsing from MTX maintenance were given a variety of reinduction and maintenance regimens. Of the 24 patients with AML, 11 were induced into remission and maintained with a variety of regimens but none received MTX. Of the 7 patients with AUL, 5 were induced into remission usually with vincristine and prednisone and 3 were maintained with Regimen D or MTX and cytosine arabinoside. Thus, of a total of 16 cases of non-ALL induced into remission, 13 did not receive MTX at any time.

RESULTS

In preliminary experiments, leukemic cell suspensions from blood and marrow were incubated without MTX for periods of 1, 2, 4, 8, 12, and 24 hr. At intervals, the TdR-3H incorporation rate was measured during a 28-min-pulse. The TdR-3H rate dropped after 4 hr incubation in some cases. Since this decline in the control rate after 4 hr could have been due to suboptimal culture conditions, we restricted all subsequent observations to the initial 4 hr of incubation. Various concentrations (1 × 10⁻⁶ M to 1 × 10⁻⁴ M) of MTX were tested for their effects on TdR-³H incorporation. In the 1st hr of incubation MTX stimulated TdR-³H incorporation, but then in some cases suppression was observed (usually only in blood blasts, not marrow blasts) and higher concentrations of MTX did not produce much more suppression of TdR-³H incorporation than 1 × 10⁻⁶ M. Hence, 1 × 10⁻⁶ M was used in subsequent studies.

Experiments were performed on the blood blasts drawn from the same patient on consecutive days before therapy. The difference (sign ignored) in percentage of stimulation or suppression between the 2 studies was calculated. From 14 such pairs the mean of the differences was derived for blood blasts and was found to be 9 ± 5.5% (S.D.). Thus, differences greater than 20% between 2 MTX-treated rates would be significant. For example, if MTX suppressed incorporation of TdR-³H to 24% of the control rate in one study and in another study suppressed incorporation of 45% of control, then the effect of the MTX in the 2 studies could be significantly different. The data from the 1st study done on each patient immediately after diagnosis and preferably before transfusions was used as that patient's in vitro response in subsequent correlations.

Data from 2 studies done on marrow specimens drawn on consecutive days and 3 duplicate studies done on the same marrow specimen on the same day were pooled. The mean of the differences was 23 ± 7.5%. There were no differences in the control (untreated) TdR-³H rates between any groups, i.e., blood versus marrow, ALL versus AML, children versus adults (data not shown).

On the average, the 4-hr in vitro treatment with MTX suppressed TdR-³H incorporation by blood blasts relative to its effect on TdR-³H incorporation by marrow blasts (Chart 1). The differences between blood and marrow blasts were most marked for lymphoblastic leukemia cells (Chart 2). Where blood and marrow blasts from the same patient were studied simultaneously, in 29 of 36 cases the blood blasts were more sensitive than the marrow blasts.

In patients with ALL there was a relationship between in vitro effect of MTX and length of remission on MTX maintenance therapy, in most cases Regimen D. In general, the patients whose lymphoblasts were most sensitive in vitro remained in remission the longest (Chart 3). Too few cases of non-ALL were maintained with MTX for analysis of their data.

Of special interest were the studies in 3 adults with AML whose blood blasts were tested at diagnosis and who then received supportive therapy only for 3 to 22 weeks until an increasing blast count and marrow failure necessitated chemotherapy. Repeat studies were done on the blood blasts just before chemotherapy. At this time, the blasts were found to be less sensitive to MTX compared to initial studies (Table 1). Patient A. C. was studied serially during the 22-week period when he received no chemotherapy. As the blood blasts increased in number, they became less sensitive to MTX in vitro (Chart 4). These observations prompted us to inspect further the relationship between the blast count in the blood and the in vitro response of the blasts to MTX.

The lower the circulating blast count of the patient at the time of testing, the greater was the in vitro suppressive effect of MTX on TdR-³H incorporation. The data for all the
MTX Effects on Acute Leukemia Blasts

Since in the present study patients with low blast counts tended to have the most suppressible blasts (Chart 5), it was of interest to see whether the blast count at diagnosis or the in vitro effect of MTX could be related to the duration of survival. For this purpose, it was necessary to restrict the analysis to only those patients who have died of their disease or survived past 36 months because the present study is ongoing and new patients are continuously being admitted into the study group. The blast count at diagnosis did or did not correlate with the duration of survival depending on whether Patient B. K. was excluded or included (Chart 6). B. K. had a high blast count at diagnosis but is still surviving 52 months later. However, there was a significant correlation between the in vitro effect of MTX and duration of survival whether B. K. was included or not because the blasts of B. K. were quite sensitive to MTX in vitro when he was first diagnosed (Chart 7). None of the data from the still-surviving patients is inconsistent with that shown in Chart 7.

Since adults with ALL have a shorter survival than do children with ALL, a comparison was made of the in vitro effect of MTX on the blasts of the 2 groups. Although there was overlap, the blasts of the children were significantly more sensitive to MTX than the blasts of the adults.

patients are shown in Chart 5. The correlation between blast count and in vitro effect of MTX was highly significant whether the data were further subgrouped according to cell type or patient age (not shown). In fact when the data were subgrouped into more homogeneous groups, the scatter diminished considerably, in the cases of ALL.

In lymphoblastic leukemia, patients with low blast counts tend to survive longer than those with high blast counts (16).

### Chart 1. Effect of MTX on blasts from blood and marrow. Cells from untreated patients were exposed to $1 \times 10^{-6}$ M MTX in vitro. After 4 hr of treatment the cells were pulsed with TdR-3H for 28 min and the rate of TdR-3H incorporation was measured and expressed as a percentage of the rate in cells not exposed to MTX. Means ± 1 S.E. are shown. The difference between the means is significant ($p < 0.001$).

### Chart 2. Effects of MTX on blasts from blood and marrow. Experimental design and expression of results as in legend to Chart 1 but patients with ALL are separated from those with AML. Differences are significant between each pair of blood and marrow and between ALL marrow and AML marrow ($p < 0.001$).

### Chart 3. Relationship between in vitro effect of MTX on ALL blood blasts at diagnosis and length of remission on MTX maintenance therapy. Cells from each patient were treated for 4 hr in vitro with $1 \times 10^{-6}$ M MTX after which they were pulsed with TdR-3H for 28 min. The rate of TdR-3H incorporation, expressed as a percentage of the rate in cells not exposed to MTX, is shown and against this is plotted the subsequent length of remission of the patient on MTX maintenance therapy, usually Regimen D (see "Materials and Methods").

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time of study</th>
<th>Blast count (per cu mm blood)</th>
<th>TdR-3H rate after MTX in vitro (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. C.</td>
<td>At diagnosis</td>
<td>700</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>22 wk later</td>
<td>17,200</td>
<td>100</td>
</tr>
<tr>
<td>M. S.</td>
<td>At diagnosis</td>
<td>240</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>3 wk later</td>
<td>975</td>
<td>93</td>
</tr>
<tr>
<td>W. G.</td>
<td>At diagnosis</td>
<td>600</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>15 wk later</td>
<td>21,000</td>
<td>85</td>
</tr>
</tbody>
</table>

a All 3 patients had AML.
Chart 4. Effect of MTX on blasts of a patient with AML. The patient was not treated for 22 weeks following diagnosis. At intervals during this time, the effect on his blood blasts of a 4-hr in vitro exposure to MTX was determined. The rate in the presence of MTX is expressed as a percentage of the rate in the absence of MTX (○), while the blast count circulating in the blood at the time the tests were done is expressed in thousands per cu mm (cmm) (*)

Chart 5. Effect of MTX on blood blasts correlated with the circulating blast count. Experimental design and expression of results as in legend to Chart 1. The rate was plotted against the absolute blast count in the blood at the time of in vitro testing. cmm, cu mm.

Chart 6. Circulating blast count at diagnosis correlated with survival in ALL. Patient B. K. is surviving over 52 months despite presenting with a high blast count. The correlation is not significant (n.s.) if Patient B. K. is included. cmm, cu mm.

Chart 7. Effect on MTX on ALL blood blasts correlated with duration of survival. Experimental design and expression of results as in legend to Chart 1. The test results at diagnosis are plotted against the survival of the patient. * Patient B. K., still surviving. The correlation is significant whether or not Patient B. K. is included.

Chart 8. Comparison of effects of MTX on ALL blood blasts of children (<15 years old) and adults. Experimental design and expression of results as in legend to Chart 1. The difference between the means is significant (p < 0.05).

Sensitive to MTX in vitro then were the blasts of adults (Chart 8). There was no difference between the blast counts at diagnosis in the 2 groups. To date, too few adults with ALL have succumbed to their disease to allow comparison of the survival between adults and children, but the 2 patients with the shortest survival in Chart 7 are both adults.

Of the 31 cases of non-ALL 6 patients did not receive any chemotherapy, 2 received ineffective doses or drugs, and 2 died of hemorrhage in the 1st 2 weeks of the induction attempt. It seemed appropriate to exclude these cases from correlation analysis because none received an adequate attempt at induction of remission. Thus the initial trial of chemotherapy was adequate in 21 cases. When the responses of blasts from those patients who received adequate therapy and who subsequently died were analyzed, there was a significant correlation between sensitivity to MTX in vitro and duration of survival (Chart 9). This was not simply because the
with folinic acid concentrations 50 to 3000 times higher (5 × 10^{-5} \text{ M} \text{ to } 3 \times 10^{-3} \text{ M}) than the MTX concentrations (Table 3). In fact, rates in the presence of folinic acid and MTX were lower than with MTX alone. Considering the lower rate with folinic acid alone, the effect of MTX was the same in the “rescued” cultures. In other experiments folinic acid, 5 × 10^{-5} \text{ M}, was added simultaneously with the MTX from the beginning of the incubation. This prevented suppression of TdR-3H by MTX, indicating that the folinic acid could at least compete with MTX before the MTX had suppressed TdR-3H incorporation (data not shown).

**DISCUSSION**

MTX is actively transported into some cells, inhibits the enzyme DHFR, and depletes cells of reduced intracellular folates. This inhibits de novo synthesis of pyrimidines and purines (1), leading to thymineless death of some S-phase cells (2) and also to purineless death of others (7). Studies of MTX transport (11) may predict which leukemic cells might be liable to its cytotoxic effect, but they do not predict the chance of inducing remission with the drug or of duration of survival. Studies of DHFR enzyme levels in blasts of patients just before they receive MTX do not predict therapeutic response, although the blasts of patients relapsing on MTX have higher levels of DHFR enzyme (9). Thus, the basis of the relative selectivity for some malignant cells in humans is not understood. The selective effect may occur because some malignant cells may traverse the cell cycle more rapidly than the S-phase leukemic cells or that the cells were alive but MTX suspensions could not prevent the suppression of TdR-3H by MTX, indicating that the folinic acid could at least compete with MTX before the MTX had suppressed TdR-3H incorporation (data not shown).

**Table 2**

<table>
<thead>
<tr>
<th>Additives</th>
<th>TdR-3H incorporation rate(^a) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
</tr>
<tr>
<td>Purines(^b) only</td>
<td>106 ± 5(^c)</td>
</tr>
<tr>
<td>MTX only</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>Purines plus MTX</td>
<td>53 ± 3</td>
</tr>
</tbody>
</table>

\(^a\) Measured after 4 hr treatment.
\(^b\) Purines added were various combinations of hypoxanthine, adenosine, guanosine, inosine, and deoxyadenosine, in concentrations of 1 × 10^{-6} \text{ M} \text{ to } 5 \times 10^{-5} \text{ M}.
\(^c\) Mean ± S.E.; 18 tests were performed on the blasts of 11 patients.

**Table 3**

<table>
<thead>
<tr>
<th>Additives</th>
<th>TdR-3H incorporation rate(^a) (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Folinic acid only</td>
<td>75 ± 13(^b)</td>
</tr>
<tr>
<td>MTX only</td>
<td>54 ± 11</td>
</tr>
<tr>
<td>MTX plus folinic acid</td>
<td>38 ± 5</td>
</tr>
</tbody>
</table>

Cells were incubated for 4 hr with 1 × 10^{-5} \text{ M} MTX, then folinic acid 5 × 10^{-5} \text{ M} to 3 × 10^{-3} \text{ M} was added. After 2 hr of “rescue,” TdR-3H incorporation rates were measured.

\(^b\) Mean ± S.E.; results are from 6 experiments (6 different patients).
do normal proliferating cells and, therefore, per unit time of treatment, more malignant cells than normal cells will be exposed to and killed by the drug. However, if anything, leukemic cells proliferate more slowly than do normal cells (3). Another possibility is that some malignant S-phase cells may be more sensitive to MTX than other S-phase cells (10). In the present study attention has been focused on the effects of MTX on DNA-synthesizing (S-phase) blasts.

The effect of MTX on deoxyuridine-3H incorporation by acute leukemia blasts after 1 hr in vitro treatment does not predict which patients will go into remission (9). Beyond 1 hr the very low incorporation rate of deoxyuridine-3H in the MTX-treated cells is difficult to measure accurately. Because of this and because of the studies in L5178Y cells, we measured the effect of MTX on TdR-3H incorporation. It soon became apparent that, compared to blood blasts, the marrow blasts were resistant to MTX in vitro. This was of interest because, in humans with acute leukemia, the blasts in the blood are much more rapidly cleared than are blasts in the marrow during chemotherapy with MTX (9). Thus, blood blasts may be more sensitive to MTX both in vitro and in vivo. However, there was no relationship between the in vitro effect of MTX on marrow blasts and individual clinical responses in the present study. Thus, attention was focused on blood blasts.

Although others (13) have reported that the labeling index of blasts at diagnosis bears on prognosis in AML and AUL, we could not relate the control rate of incorporation of TdR-3H of the blasts to the duration of survival. The degree of periodic acid-Schiff staining in the ALL patients did not bear on prognosis in our hands (12). We did not study the relationship between marrow blast size and prognosis (14).

Herbert et al. (6) observed that MTX suppressed TdR-3H incorporation in some tumor cells but not normal marrow cells and suggested that such a test system might allow for selection of chemotherapeutic agents for clinical use.

In the present study, MTX suppressed TdR-3H incorporation in the blood blasts from some cases. The lower the blast count in vivo, the more sensitive were they to MTX in vitro and the better did the patients fare. The differences between a "sensitive" and a "resistant" case were not great in linear terms, e.g., a MTX rate of 33% of control in a sensitive case versus 133% in a resistant case. However, incorporation was measured as a rate (intermittent labeling during a 28-min pulse period; cf. Ref. 9), and the error of measurement was kept below a fixed minimum percentage of the rate. Thus it was possible to analyze the data in the logarithmic form (15), in which the difference between the cases cited above would be quite significant, i.e., 4-fold or 0.6 log. The data correlating in vitro effect with duration of remission or survival are presented in the form of double-log plots. This format minimizes the appearance of individual variability. However, in a biological system such as the present one where relationships are probably multifactorial and where the variables cover a wide range, the double-log format is necessary before one can quantitate relationships at both extremes of response. Thus, for example, in Charts 7 and 9 the relationship is statistically significant, although individual variation was considerable. Follow-up of a larger number of patients will be required to determine the extent to which the TdR-3H suppression test is of practical value in predicting duration of remission or survival in individual cases.

Patients with ALL with low blast counts frequently do much better than patients with high blast counts (16). However, as a prognostic index, measurement of the in vitro effect of MTX on TdR-3H incorporation by blood blasts may have advantages over a simple recording of the blast count at diagnosis, although the biochemical test is more difficult to perform. Firstly, in the present study group the biochemical test was a better predictor of survival than the blast count. Secondly, it discriminated to some extent between the blasts of adults with ALL and the blasts of children with ALL; those of the children were more sensitive in vitro. It is known that children with ALL tend to have a better prognosis than do adults (5).

Thirdly, in patients with non-ALL, the blast count does not bear a relationship to prognosis; whereas with the in vitro suppression test, it seems that those patients with blasts initially most sensitive in vitro may survive the longest, although the test is of limited value in cases with responses in the midrange.

Finally, the most important advantage of the biochemical test over the blast count at diagnosis is that more certain identification of "sensitive" cells by the biochemical test allows one to investigate the reasons why some cells are sensitive to chemotherapy and some are not. This may prove to be its most useful application.

The suppression of TdR-3H incorporation observed here could not be prevented by purines or reversed by delayed addition of folinic acid. Thus, suppression of TdR-3H incorporation may have been due to kill of S-phase cells rather than to induction of a purineless state as occurs early during treatment of L5178Y lymphoblasts. However, it could also have been due to some other mode of action of MTX on DNA synthesis. Our data do not allow us to choose between these alternatives.

It is not clear why there are differences in responses of blasts from blood versus marrow, especially in ALL, from low-white-count leukemias versus those with a high white count, or from children with ALL versus adults with ALL. Studies of MTX transport, DHFR levels, or reduced folate pools might explain these differences.

Regardless of the reasons for the differences it seems clear that others doing biochemical or immunological studies on leukemic blasts would do well to take into account the age of the patient if the disease is ALL, the compartment from which the cells are sampled, and, if blood blasts are studied, the level of the circulating blast count at the time the cells are sampled.

ACKNOWLEDGMENTS

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