The Reduction of Folate and of Dihydrofolate by Homogenates of Leukocytes from Patients with Leukemia or with Myeloid Metaplasia

DeWAYNE ROBERTS AND THOMAS C. HALL

Laboratories of Pharmacology, Children's Cancer Research Foundation, and Departments of Pathology and Medicine, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

Homogenates of human leukocytes reduced dihydrofolate more rapidly than folate. A correlation was observed between the assay of the enzyme with folate and with dihydrofolate. Homogenates of cells from patients receiving methotrexate reduced dihydrofolate much more rapidly than folate. Uninhibited enzyme was observed with both substrates in some homogenates of cells from patients receiving methotrexate. The level of enzyme activity in chronic myelogenous leukemia was correlated with the white blood cell count of the patient. Low levels of enzyme activity were observed in leukocytes from patients with myeloid metaplasia.

INTRODUCTION

The inhibition of dihydrofolate reductase has been suggested as the pharmacologic basis of the chemotherapeutic action of methotrexate in the treatment of acute leukemia (1, 24). Purified preparations of this enzyme from avian, bacterial, and animal sources were inhibited by very low concentrations of methotrexate (3, 16, 19, 21, 25, 32). The administration of methotrexate to rodents bearing transplantable leukemias resulted in a decrease in enzyme activity in the tumor cells (28). However, an increase in the level of enzyme activity, rather than a decrease, was observed in white blood cells from patients with leukemia following the administration of methotrexate (3–5). In the present work, the basis of this anomalous elevation of the activity of the target enzyme has been reexamined in human white blood cells.

In acute myelogenous, acute lymphocytic, and chronic myelogenous leukemia, dihydrofolate reductase activity of human leukocytes is elevated above normal levels (5, 6, 7, 20, 22, 32). A drug-induced elevation of enzyme activity was also observed in patients responding to methotrexate. Dihydrofolate was used as substrate at pH 8.3 in these studies on the "induction" of dihydrofolate reductase from human leukocytes. With enzyme from mouse ascites tumor, these conditions led to a more effective competition by dihydrofolate for the drug for the enzyme (2). The higher enzyme levels reported in the cells of patients receiving methotrexate would be difficult to interpret accurately because of the competition between methotrexate and dihydrofolate, which would result in the release of a portion of in vivo drug-bound enzyme.

Highly purified preparations of dihydrofolate reductase reduced folate and dihydrofolate to tetrahydrofolate (2, 16, 19, 21, 25, 32). The ability of the enzyme to reduce two substrates has led to the development of assays with folate and with dihydrofolate (11, 23). Differences have been noted in the characteristics of the inhibition of dihydrofolate reductase by methotrexate with the two substrates (2, 19, 25, 26). In the presence of dihydrofolate, the enzyme was less effectively inhibited by methotrexate than with folate as substrate (2, 17, 25). With folate as substrate, the enzyme activity could be stoichiometrically inhibited by the addition of graded amounts of methotrexate (29). Dihydrofolate reductase had a lower Michaelis constant for dihydrofolate than for folate, which contributed to a more effective competition by dihydrofolate for the enzyme in the presence of the drug (25). In addition, the assay with folate was made at a lower pH, usually pH 6.1, than most workers used with dihydrofolate as substrate. The more alkaline pH used in the assays with dihydrofolate was shown to favor the dissociation of the drug-enzyme complex (2). The correlation between the assay of dihydrofolate reductase with folate and with dihydrofolate as substrates is reported here. A preliminary report of this work has been presented (22).

MATERIALS AND METHODS

Isolation of Human White Blood Cells

The blood was collected in 10-ml Vacutainers containing 10 mg disodium ethylenediaminetetraacetate, immediately chilled, and kept at 4°C throughout the isolation of the leukocytes. Sedimentation of erythrocytes was accelerated by the addition of an equal volume of 0.9% NaCl solution containing either 6% bovine fibrinogen or 3% dextran, M.W. 250,000 (24), and the leukocytes were isolated by the method of Fallon et al. (9). In our studies, no difference was noted when dextran was substituted for fibrinogen. The dextran was less expensive and minimized the possibility that incomplete washing might carry over some of the fibrinogen to interfere with the protein analysis. The pellet of isolated leukocytes was stored at −20°C.

Preparation of Homogenate

After thawing the pellet of white blood cells, either by warming in the hand or by allowing to stand on ice, an equal volume of 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.0, was
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added, and the cell fragments were homogenized with a poly-
ethylene pestle in a glass homogenizer. An aliquot was removed
for protein determination by the method of Lowry et al. (17).
The homogenate was centrifuged at 20,000 to 30,000 \( \times g \) for 25
minutes, and the supernatant fluid assayed for dihydrofolate
reductase activity by two methods.

**Dihydrofolate Reductase Assays**

1. **Folate Assay.** With folate as substrate, the diazotizable
amine method was used to measure the formation of tetrahy-
drofolate (23, 29). The final concentration of substrates in theassay was 1.4 \( \times 10^{-4} \) M folate and 2 \( \times 10^{-4} \) M nicotinamide
adenine dinucleotide phosphate reduced (NADPH) (23). As a
control for this assay the homogenates were incubated with the
complete system plus methotrexate, 40 m\( \mu \)g/ml. The enzyme
reaction with homogenates of human white blood cells was
linear with time for two hours and proportional to the concen-
tration of the homogenate.

2. **Dihydrofolate Assay.** With dihydrofolate as substrate,
the tetrahydrofolate formed was converted to \( \text{N}^6,\text{N}'^{10} \)-formyltetra-
hydrofolate. The \( \text{N}^6,\text{N}'^{10} \)-formyltetrahydrofolate was converted to
\( \text{N}^6,\text{N}'^{10} \)-methyltetrahydrofolate by the addition of trichloro-
acetic acid. The homogenate was supplemented with
formyltetrahydrofolate synthetase (EC 6.3.4.3), formate, and
adenosine triphosphate (6). The dihydrofolate was prepared by
dithionate reduction of folate in the presence of mercaptoethanol
(12).

**RESULTS**

**Methotrexate Titration of Enzyme Activity**

With folate as substrate, the addition of graded amounts of
methotrexate stoichiometrically inhibited the enzyme. By meas-
uring the endpoint of this titration of enzyme activity, the
enzyme content could be reported in terms of moles of metho-
trexate required to inhibit the enzyme completely. Chart 1
presents an example of the titration of dihydrofolate reductase
from white blood cells of an untreated patient with acute lympho-
cytic leukemia. The measurement of dihydrofolate reductase by
titration of enzyme activity has the advantage of being much
more independent of changes in the concentration of NADPH
than the measurement of the amount of product formed (23).
However, this method requires a large volume of white blood
cells, which was frequently not available from pediatric patients
or from patients with a low white blood cell count.

**Assay of Dihydrofolate Reductase with Dihydrofolate**

The requirements for the assay of dihydrofolate reductase
from white blood cells with dihydrofolate as substrate in the
coupled enzyme assay are presented in Table 1. The concentra-

![Chart 1. Methotrexate inhibition of the reduction of dihydro-
folate. The conditions of the assay were similar to those in Table 1
except that graded amounts of methotrexate were added.](chart)

![Chart 2. Methotrexate inhibition of the reduction of dihydro-
folate. The conditions of the assay were similar to those in Table 1
except that graded amounts of methotrexate were added.](chart)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Absorbancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Complete</td>
<td>0.874</td>
</tr>
<tr>
<td>2. Complete + MTX (9 ( \times 10^{-7} ) M)</td>
<td>0.351</td>
</tr>
<tr>
<td>3. - NADPH</td>
<td>0.370</td>
</tr>
<tr>
<td>4. - DHFA</td>
<td>0.339</td>
</tr>
<tr>
<td>5. - ATP</td>
<td>0.202</td>
</tr>
<tr>
<td>6. - Formate</td>
<td>0.220</td>
</tr>
<tr>
<td>7. - Formate-activating enzyme</td>
<td>0.235</td>
</tr>
</tbody>
</table>

* Fifty \( \mu \)l of an homogenate of white blood cells from a patient
with acute lymphocytic leukemia were incubated in a final volume
of 250 \( \mu \)l containing 1 \( \times 10^{-4} \) M DHFA, 3.5 \( \times 10^{-4} \) M NADPH,
0.005 M ATP, 0.05 M sodium formate, 0.02 M NaF, 0.01 M mercap-
toethanol, 0.05 M NH₄Cl, 0.02 M MgCl₂, and 0.15 M tri(hydroxy-
methyl)aminomethane, pH 8.3, for 30 minutes at 37°C (6). The
system was supplemented with a purified preparation of formyl
tetrahydrofolate synthetase from chicken liver. The \( \text{N}^6,\text{N}'^{10} \)-formyl-
tetrahydrofolate was converted to \( \text{N}^6,\text{N}'^{10} \)-methyltetrahydrofolate
with the addition of 100 \( \mu \)l 20% trichloroacetic acid, and the ab-
sorbancy measured at \( \lambda = 355 \) nm.

\( \text{MTX}, \) methotrexate; \( \text{NADPH}, \) nicotinamide adenine di-
nucleotide phosphate, reduced; \( \text{DHFA}, \) dihydrofolate; \( \text{ATP}, \)
adenosine triphosphate.
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**CHART 3.** Measurement of dihydrofolate reductase by reduction of folate versus methotrexate titration of enzyme activity. Homogenates of white blood cells were inhibited by the addition of graded amounts of methotrexate as in Chart 1. The amount of drug required to inhibit the enzyme completely was plotted against the amount of tetrahydrofolate formed in the absence of the drug. Homogenates of leukocytes were prepared from patients with acute lymphocytic leukemia (ALL), O; acute myelogenous leukemia (AML), □; chronic myelogenous leukemia (CML), X; and chronic lymphocytic leukemia (CLL), △. THFA, tetrahydrofolate.

Measurements of substrate and cofactors reported by Bertino et al. (6) were confirmed as optimal for this enzyme. The conversion of dihydrofolate to tetrahydrofolate results in a decrease in the absorbancy at λ = 355 μm (11). As a result of the reduction of dihydrofolate to tetrahydrofolate, the omission of formate from the controls, as suggested by the original workers (Dr. J. R. Bertino, personal communication), resulted in a high estimate of enzyme activity by giving a low value for the blank. Condition 2 of Table 1 was used in the present work as a control to correct for the absorbancy of the reagents and homogenate. The rate of the reaction was linear for 30 minutes at 37°C and was proportional to the concentration of white blood cell homogenate.

**Methotrexate Inhibition of Enzyme Activity in the Presence of Dihydrofolate**

When graded amounts of methotrexate were added to a homogenate of white blood cells and the enzyme activity was measured with dihydrofolate by the formation of N-formyltetrahydrofolate, much higher levels of drug were required to inhibit the enzyme than when dihydrofolate was used as the substrate (Chart 2). The more alkaline conditions used in the assay with dihydrofolate, as well as the use of dihydrofolate as substrate, apparently resulted in a more competitive relationship with the drug and the enzyme.

**Survey of White Blood Cells**

Dihydrofolate reductase has been assayed in white blood cells from patients with leukemia or myeloid metaplasia. The level of enzyme activity in normal leukocytes and in leukocytes from patients with chronic lymphocytic leukemia was below the sensitivity of the assay with folate. The elevated dihydrofolate reductase activity in white blood cells from patients with acute leukemia or chronic myelogenous leukemia with dihydrofolate as substrate (1, 20, 31) was also observed with folate as substrate.

**CHART 4.** Correlation between the measurement of dihydrofolate reductase with folate and dihydrofolate with homogenates of white blood cells from patients with acute lymphocytic leukemia (ALL), O; or acute myelogenous leukemia (AML) □. The standard deviation of the slope of the regression line was ±0.048.
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Chart 5. Correlation between the reduction of dihydrofolate and folate with homogenates of leukocytes from patients with chronic myelogenous leukocytes.

Assay of Dihydrofolate Reductase with Two Substrates

Homogenates of white blood cells from various leukemic types have been assayed with folate and with dihydrofolate as substrate (Charts 4, 5). A high correlation was observed between the two assays, unless the patient was receiving methotrexate at the time that the blood was drawn. The coefficient of correlation between the assays of cells from patients not on methotrexate was 0.68 and 0.74 (Charts 4, 5). White blood cells from patients receiving methotrexate showed much higher levels of enzyme activity with dihydrofolate than with folate as substrate (Chart 6). The coefficient of correlation between the assays was 0.66.

Correlation between the White Blood Cell Count and the Level of Dihydrofolate Reductase Activity

Chart 7 presents the correlation between the white blood cell count of the blood and the level of dihydrofolate reductase activity, measured with dihydrofolate, in cells from patients with chronic myelogenous leukemia. As the cell count increased, inhibit enzyme activity completely. The results obtained with homogenates of white blood cells from patients with acute leukemia or chronic leukemia are included together. The high level of enzyme activity observed in this figure with one patient with chronic lymphocytic leukemia has been observed only one other time. The equation for the line was \( y = 0.32x + 170 \) (30). The dotted lines indicate the 95% confidence levels for the slope of the regression line (18). The coefficient of correlation between the two assays was 0.72 (13). The coefficient of correlation between the assays suggests that dihydrofolate reductase can be measured with confidence by the amount of product formed. The turnover number of approximately 10 \( \mu \)moles/min/mole methotrexate was in the range reported earlier by Werkheiser with enzyme preparations from rodents (26).
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the level of reductase activity also increased. The coefficient of correlation between these two parameters was 0.61.

No significant correlation, \( r = -0.18 \), was observed between the level of dihydrofolate reductase and the white blood cell count in myeloid metaplasia (Chart 8). The enzyme activity was lower than the level observed in white blood cells from patients with chronic myelogenous leukemia.

**DISCUSSION**

Dihydrofolate reductase from human leukocytes has been assayed with folate and with dihydrofolate as substrates. Stoichiometric inhibition by methotrexate was observed with folate as the substrate, and a correlation was observed between the measurement of enzyme activity by methotrexate titration and by the reduction of folate to tetrahydrofolate. The low levels of enzyme activity present in white blood cells and the difficulty of obtaining an adequate number of cells made a more sensitive assay desirable.

Conditions for the assay in which dihydrofolate was converted to tetrahydrofolate were studied. A strong correlation was observed between the assay with folate and the \( N^\text{5,7} \)-formyltetrahydrofolate assay with dihydrofolate, unless the leukocytes were obtained from patients who were receiving methotrexate. White blood cells from these patients had much higher levels of enzyme activity with dihydrofolate as substrate than with folate. Analysis of the t test for the standard deviation of the slopes of the regression lines in Charts 4 and 6 gives a value, 0.05 > \( P > 0.02 \), such that the data in these charts could have come from the same population. Examination of the inhibition of dihydrofolate reductase by methotrexate indicated that methotrexate was not a stoichiometric inhibitor of the enzyme under the conditions of the assay with dihydrofolate. In relation to the in vivo conditions, an increase in the drug-enzyme complex may have occurred under the conditions of the assay with folate or dissociated under the conditions of the assay with dihydrofolate.

Methotrexate is retained for long periods of time in the human (8) and in the rodent (10, 27). Retention of the drug was attributed to binding to dihydrofolate reductase (26). Although the drug may reside much of the time in a complex with the enzyme, labeled methotrexate can be flushed from the human with unlabeled drug or by \( N^\text{5,7} \)-formyltetrahydrofolate (14).

These observations suggest that in vivo the drug is in equilibrium with the enzyme, but that the level of free drug may be so low that effective clearance of the drug from the cell does not occur. Synthesis of new enzyme would favor the formation of the drug-enzyme complex and prolonged retention of the drug. This would explain the basis of the prolonged retention of methotrexate and could favor the formation of additional drug-enzyme complex under the conditions of the assay with folate.

Dissociation of the drug-enzyme complex would occur under the conditions of the assay with dihydrofolate. When the assay with dihydrofolate was applied to the study of enzyme from human white blood cells, an increase in enzyme activity was observed with methotrexate therapy (1, 3-5). This enzyme "induction" was noted in white blood cells from patients with acute leukemia who, with continued therapy with the drug, went into remission (1, 3-5). If the pharmacologic action of methotrexate results from an inhibition of dihydrofolate reductase, then the anomalous increase in enzyme activity observed in vitro may result from a dissociation of the drug-enzyme complex, or from some other factor, such as two different enzymes, or from a combination of these possibilities. The failure of the regression lines to pass through zero or of the 95% confidence range for the slopes of the regression lines to include zero could be explained on the basis of two enzymes, only one of which reduced folate (15). The increase in the enzymatic capacity of the cells to reduce dihydrofolate following the administration of methotrexate would then result from an increase in the activity. Or, since the methods are being applied close to the limits of their sensitivity, the failure of the regression lines to pass through zero may result from experimental variation in the assays.

Apparently, uninhibited enzyme does exist in white blood cells obtained from patients who were receiving methotrexate. This conclusion is suggested by the observation of enzyme activity by the folate assay with these cells (Chart 6). The relationship between the enzyme level, the course of the disease, and response to methotrexate must be further examined if the hypothesis is
to be substantiated that the pharmacologic action of methotrexate results from the inhibition of dihydrofolate reductase.

The difference observed in the level of dihydrofolate reductase in leukocytes in myeloid metaplasia and chronic myelogenous leukemia may offer another biochemical parameter for distinguishing between these two diseases. The greatest difference in enzyme level between chronic myelogenous leukemia and myeloid metaplasia was observed with cells from patients with high leukocyte count. Further study is needed before it will be known if the enzyme level in a patient with myeloid metaplasia can be correlated with a progression toward chronic myelogenous leukemia.

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REFERENCES

18. Mainland, D. Notes from a Laboratory of Medical Statistics. New York University College of Medicine, New York, New York.
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