On the Poor Correlation between the Inhibition by Methotrexate of Dihydrofolate Reductase and of Deoxynucleoside Incorporation into DNA

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SUMMARY

Inhibition by Methotrexate of dihydrofolate reductase and of the incorporation of deoxynucleosides into DNA has been examined in three mouse ascites cell leukemias which had differences in uptake and sensitivity. Dihydrofolate reductase activity in all cell lines was completely inhibited in less than five minutes after the optimal therapeutic dose of Methotrexate. The rate of recovery of enzyme activity was not related to the effectiveness of the drug in prolonging the life of the host. Methotrexate blocked the in vivo incorporation of deoxyuridine into DNA in the sensitive line and increased the utilization of thymidine without provoking a statistically significant change in the drug-induced resistant or the innately insensitive lines. Although dihydrofolate reductase activity was severely inhibited in cells from Methotrexate-treated animals, the in vitro incorporation of deoxyuridine into DNA was less effectively inhibited than in vivo. The addition of Methotrexate in vitro to cells from Methotrexate-treated animals increased the inhibition of deoxyuridine incorporation in the sensitive and innately insensitive cell lines. Although dihydrofolate reductase had been inhibited in vivo by the drug, Methotrexate concentrations in excess of $3 \times 10^{-5} \text{ M}$ were required in vitro to inhibit deoxyuridine incorporation by the resistant cells. Presumably, Methotrexate not bound to the enzyme was washed from the cells during isolation. To achieve in vitro an inhibition of deoxyuridine similar to that observed in vivo after treatment with Methotrexate required an excess of drug beyond that bound to the enzyme.

INTRODUCTION

The inhibition of dihydrofolate reductase has been suggested as the basis of the pharmacologic action of Methotrexate (1, 24). This enzyme catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate, which participates in a number of reactions requiring "one-carbon" units (9, 15). In the absence of dihydrofolate reductase activity, the oxidation of tetrahydrofolate, as in the conversion of deoxyuridine 5'-monophosphate to thymidine 5'-monophosphate, would lead to depletion of tetrahydrofolate cofactors. Wells and Winzler observed that in human leukocytes the synthesis of DNA was more sensitive than RNA synthesis to inhibition by Methotrexate (21, 25). This observation suggested, as one possibility, that Methotrexate was exerting a selective inhibition of thymine biosynthesis, the principally labeled constituent of DNA in their studies.

Resistance to Methotrexate, developed by the constant exposure of cell cultures to low levels of the drug, was correlated with an increase in dihydrofolate reductase activity (8, 12) and led to the proposal that enzyme synthesis exceeded the rate of Methotrexate uptake by these cells (11). An increased level of dihydrofolate reductase was also observed in transplantable mouse leukemic cells in which resistance to Methotrexate had been developed (3, 10), but no correlation was observed between the level of dihydrofolate reductase and the response to Methotrexate of transplantable mouse leukemias not exposed to the drug prior to the testing of their sensitivity (20). A correlation was, however, observed between the rate of Methotrexate uptake by ascites cells in vitro and the prolongation by the drug of the life of the tumor-bearing hosts (13).

Impaired uptake could result in an insignificant inhibition of dihydrofolate reductase or could allow a more rapid recovery of dihydrofolate reductase after the major fraction of the administered drug had been excreted and the drug level in the extracellular space has fallen to ineffective levels. When the level of tetrahydrofolate cofactors becomes limiting as a result of the inhibition of dihydrofolate reductase, the incorporation of deoxyuridine into DNA should be blocked, and if there is a selective inhibition of the methylation of deoxyuridine 5'-monophosphate, thymidine incorporation should be stimulated.

The present study examines the relationship between the inhibition of dihydrofolate reductase in tumor cells following Methotrexate administration to the host and the incorporation into DNA of thymidine and of deoxyuridine which must be converted to thymidine derivatives. These parameters were
studies in a Methotrexate-sensitive mouse leukemia, L1210; a
drug-induced resistant variant, L1210/MTX; and an innately
resistant cell line, P329.

MATERIALS AND METHODS

Isolation of the Ascites Tumor Cells. One million ascites tu-
mor cells were inoculated intraperitoneally into BDF1 mice (20).
These animals were used for studies beginning on Day 5
with L1210, Day 6 with L1210/MTX, or Day 9 or 11 with
P329. The animals were killed by cervical dislocation. The ascites
cells were placed in five ml of solution containing sodium ethy-
lenediaminetetraacetate (EDTA), 1 mg/ml, and 5% dextrose
at pH 7.4. The temperature was held between 0°C-4°C during
isolation of the cells by a modification of the method of Fallon
et al. (7), in which the erythrocytes were removed by hypotonic
shock for 25 sec. The cells were repeatedly washed with 0.9%
NaCl, which would have diluted the extracellular drug concen-
tration by a factor of 5 × 10⁻⁵. The isolated cells were suspen-
ded in 0.9% NaCl and divided into two aliquots. The cells in both aliquots were collected by
centrifugation. A volume of 0.01 M Tris(hydroxymethyl)aminomethane-HCl (Tris), pH
7.0, equal to the volume of the pellet, was added to one aliquot,
and the cells were disrupted by a 5-second pulse of ultra-high
frequency sound, Sonifier™ Branson Instruments Incorporated.
A fraction of the homogenate was taken for assay of the
protein content (14), and the remainder was centrifuged at
20,000 × g for 25 minutes. The supernatant fluid was stored
at −20°C for assay of dihydrofolate reductase activity.

The second aliquot of whole cells was washed with the in-
oculation medium and resuspended at the desired concentration
in incubation medium at pH 7.4, which was composed of 60%
Eagle's minimal essential medium, 25% horse serum, and 0.03
m sodium phosphate buffer. A 5% suspension by weight of wet
cells was used for studies of L1210 and L1210/MTX and a
2% cell suspension with P329.

Whole Cell Studies. Aliquots of the whole cell suspension,
140 µl, were incubated for 30 min with either 2 × 10⁻⁵ M
thymidine-methyl-³H, 1.5 × 10⁶ cpm, or 3.6 × 10⁻⁶ M de-
oxyuridine-₆⁻H, 1.2 × 10⁶ cpm, in a total volume of 150 µl.
A shaking incubator, Lab-Line Instruments, Inc., at 37°C was
used with 10 × 75 mm glass tubes for incubation. After a brief
initial lag of 2 to 3 min, the incorporation of radioactivity into
the thymine fraction of the supernatant fluid was measured by aliquoting on Whatman No. 1 paper with isopropanol-HCl (26).

Activity following Methotrexate

Enzyme activity is reported as cpm/mg protein or µmoles
tetrahydrofolate/hr/mg protein. Under these conditions a
stoichiometric inhibition of dihydrofolate reductase was ob-
served with the addition of Methotrexate. The supernatant
fluid from the sonically disrupted cells was diluted with 0.01 M
Tris, pH 7.0, to give a concentration equivalent to a 3.5%
suspension of the cell pellet. The assay was similar to that
described with rat liver (17), and the rate of folate reduction
was linear with time for at least 60 min and was proportional
to homogenate concentration at the dilutions which were used.
This assay for dihydrofolate reductase was selected for the
present studies because of problems encountered earlier when this enzyme was measured with leukocytes from patients receiv-
ing Methotrexate (18).

Methotrexate Administration. Methotrexate was adjusted to
approximately pH 8 with NaOH to bring it into solution and
diluted to the final concentration in 0.9% NaCl. One-tenth
ml of drug was administered intraperitoneally for each ten
grams body weight. Control mice received 0.9% NaCl.

RESULTS

Rate of Recovery of Dihydrofolate Reductase

Methotrexate was administered intraperitoneally to mice
bearing transplantable lymphoblastic ascites tumors, and the
extent of inhibition and rate of recovery of dihydrofolate re-
ductase activity in the ascites cells was determined (Chart 1).
One hour after the administration of 0.2 mg Methotrexate/kg,
a therapeutically ineffective dose when administered once daily,
a very pronounced inhibition of enzyme activity was observed.
Enzyme activity was recovered at a linear rate. One-third of
the original activity was present 24 hours after drug adminis-
tration.

The optimal daily, intraperitoneal dose of Methotrexate for
the treatment of BDf1 mice bearing L1210 is 1.5 mg/kg (I. Wodinsky, unpublished observation). Complete inhibition of
enzyme activity was observed at one hour and at four hours
after drug administration. Ninety-five percent of the original
enzyme activity remained inhibited after 24 hours.

The intraperitoneal administration of 1.5 mg of Methotrex-
ate/kg to mice bearing L1210/MTX completely inhibited dihy-
drofolate reductase activity at one hour after drug administra-

8 Liquid scintillation counting medium was prepared with 4 gm
of 2,5-bis[2-(5-tert-butylbenzoazosoly)]-thiophene in 600 ml tolu-
ene, 400 ml ethylene glycol monomethyl ether, with 80 gm naphthy-
lene according to the instructions of Packard Instrument Com-
pany, Inc.

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Chart 1. The inhibition and subsequent recovery of dihydrofolate reductase activity following the intraperitoneal administration of Methotrexate. Mice bearing L1210 received either 0.2 or 1.5 mg of Methotrexate/kg, •—•. Mice bearing L1210/MTX received 1.5 mg of Methotrexate/kg, •—•; saline controls, ○—○. The dihydrofolate reductase activity was assayed as described earlier (17). 20.4 μl of supernatant fluid of a 3.5% dilution of mouse ascites tumor cells was incubated for forty minutes at 37°C with 4.53 μl reagent containing 3,3-dimethylglutarate, citrate, MgCl₂, nicotinamide adenine dinucleotide phosphate (reduced), and folate-G-³H at pH 6.1. The reaction was terminated with the addition of trichloroacetic acid containing KNO₂, and the residual substrate absorbed onto charcoal. An aliquot of the supernatant was removed for counting by liquid scintillation technics. MTX, Methotrexate.

Dihydrofolate Reductase Activity and DNA Labeling

The Relationship between Methotrexate Inhibition of Dihydrofolate Reductase and Deoxynucleoside Incorporation into DNA. The inhibition of dihydrofolate reductase activity presumably blocks the reduction of folate and dihydrofolate to tetrahydrofolate. The only presently known metabolic reaction in which tetrahydrofolate derivatives are oxidized is the methylation of deoxyuridine 5'-monophosphate to form thymidine 5'-monophosphate (Chart 3). This reaction is catalyzed by thymidylate synthetase and results in the conversion of N⁵,N¹⁰-methylene tetrahydrofolate to dihydrofolate (10). The inhibition of dihydrofolate reductase should block the de novo synthesis of thymidine 5'-monophosphate and results in a stimulation of the incorporation of exogenous thymidine into DNA while inhibiting the incorporation of exogenous deoxyuridine. The first step involved in the utilization of these deoxyribonucleosides for DNA synthesis following their uptake by the cell is the conversion by thymidine kinase to their respective nucleoside 5'-phosphate (4). If the decrease in tetrahydrofolate results in a general inhibition of “one-carbon” metabolism, any increased utilization of exogenous thymidine may be masked.

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after drug administration the cells were collected and dihydrofolate reductase activity and the in vitro capacity of the cells to incorporate deoxyuridine or thymidine into DNA were measured. The doses of Methotrexate were 0.15, 0.75, 1.5, 15, and 150 mg/kg. One hour after the administration of Methotrexate, dihydrofolate reductase activity was severely inhibited by all doses of Methotrexate (Chart 4). A dose-related inhibition of deoxyuridine incorporation occurred but did not exceed fifty percent. No significant change was observed in thymidine incorporation. Twenty-four hours after drug administration, a slight recovery of dihydrofolate reductase activity was observed which appeared to be dose related. The inhibition of deoxyuridine incorporation had disappeared by then except at a dose of 150 mg/kg. At this dose of Methotrexate, which is in excess of the LD50 level, an inhibition of deoxyuridine and of thymidine incorporation into DNA was observed.

In similar studies with L1210/MTX (Chart 5), the lowest dose of Methotrexate, 0.15 mg/kg, resulted in a fifty-percent inhibition of dihydrofolate reductase activity at one hour after drug administration. A more pronounced inhibition of enzyme activity was observed at the other dose levels. At this time no effect was observed on deoxyuridine or thymidine incorporation except for a stimulation of thymidine utilization at the dose of 150 mg/kg. Enzyme activity was recovered at twenty-four hours and was dose related. No pronounced changes in the utilization of exogenous nucleosides were noted.

Chart 4. The incorporation in vitro of deoxyuridine and thymidine into DNA and the level of dihydrofolate reductase activity following the administration of various levels of Methotrexate to mice bearing L1210. The animals were sacrificed one hour or twenty-four hours after drug administration, and cells from three animals were pooled. Two groups of pooled cells were individually analyzed, and the individual values are indicated by the small horizontal line. THFA, tetrahydrofolate.

Chart 5. The effect of various levels of Methotrexate on the in vitro incorporation of deoxyuridine and thymidine into DNA and on dihydrofolate reductase activity of L1210/MTX. The drug was administered to the animals, and the cells collected and pooled as described for Chart 4. MTX, Methotrexate; THFA, tetrahydrofolate.
The pattern observed in these three parameters with P329 differed from L1210 and L1210/MTX (Chart 6). Dihydrofolate reductase was as sensitive as in L1210 to inhibition by Methotrexate and returned more slowly than with L1210. The suggestion of a dose-related inhibition of deoxyuridine incorporation was observed at one hour together with a stimulation of thymidine utilization at the highest dose of Methotrexate. At twenty-four hours the inhibition of nucleoside incorporation at 150 mg/kg was similar to that observed with L1210 (Chart 4).

The Effect of Methotrexate on the in Vivo Incorporation of Deoxyuridine and Thymidine into DNA. Three hours after administration of Methotrexate, 1.5 mg/kg, intraperitoneally to mice bearing L1210, L1210/MTX, or P329, deoxyuridine or thymidine was injected into the peritoneal cavity. The ascites cells were collected fifteen minutes after nucleoside administration and isolated as described in the Legend. The incorporation of deoxyuridine into DNA by L1210 was inhibited 80% following the administration of Methotrexate, \( p < 0.001 \) (Chart 7). The utilization of exogenous thymidine was stimulated approximately 60% following the administration of Methotrexate to mice bearing L1210, \( p < 0.05 \). Under these conditions Methotrexate did not affect the utilization of deoxyuridine or thymidine by L1210/MTX, \( p > 0.9 \). No statistically significant change in the utilization of deoxycytosines was observed with P329 following the administration of Methotrexate.

The Effect on Deoxyuridine Incorporation into DNA of the in Vitro Addition of Methotrexate. The observation of a more pronounced inhibition by Methotrexate in vivo than in vitro of the incorporation of deoxyuridine into DNA could indicate that although dihydrofolate reductase was inhibited, the maximum inhibition of deoxyuridine incorporation may require drug concentrations in excess of those required to inhibit dihydrofolate reductase. Ascites tumor cells were isolated from mice bearing L1210, L1210/MTX, or P329 three hours after the intraperitoneal administration of Methotrexate, 1.5 mg/kg, or 0.9% NaCl. This appeared from Chart 1 to be the longest period of time that could be allowed for exhaustion of endogenous tetrahydrofolate derivatives before the recovery of traces of dihydrofolate reductase activity in L1210/MTX. The in vitro incorporation of deoxyuridine into DNA by intact ascites cells was studied in the presence of various concentrations of Methotrexate (Chart 8). As the concentration of Methotrexate was increased, a more pronounced inhibition of the incorporation of deoxyuridine occurred in L1210 and in P329. A log-dose relationship was observed, with parallel lines for the in vivo control and Methotrexate-treated cells. Methotrexate at \( 3.5 \times 10^{-8} \) M did not inhibit the incorporation of deoxyuridine by L1210/MTX.

The effect of Methotrexate on the incorporation of deoxyuridine, thymidine, and deoxycytidine was studied with ascites tumor cells from saline and Methotrexate-treated animals. Methotrexate was added in vitro as indicated in Chart 9. With cells from L1210 and P329, the in vitro concentration of Methotrexate was \( 1.4 \times 10^{-6} \) M. With L1210/MTX the concentration of Methotrexate was \( 1.4 \times 10^{-4} \) M. Each experiment consisted of five replicates of cells pooled from 4 or 5 animals for each point, and the variation from the mean for the replicates was 5 percent or less. Deoxycytidine was more effectively incorporated into DNA than either deoxyuridine or thymidine. The
METHOTREXATE INHIBITION OF DEOXYURIDINE INCORPORATION INTO DNA

Chart 8. The inhibition of the incorporation of deoxyuridine into DNA by Methotrexate added in vitro to ascites tumor cells from Methotrexate- or saline-treated animals. Methotrexate, 1.5 mg/kg, or 0.9% NaCl was administered intraperitoneally to animals bearing L1210, L1210/MTX, or P329, and three hours later the cells were removed and isolated. Various levels of Methotrexate were added to aliquots of the cell suspension, and the incorporation of deoxyuridine was assayed. MTX, Methotrexate.

A more pronounced inhibition of deoxycytidine incorporation was observed with L1210 than with P329 when Methotrexate was added in vitro to cells from the saline-treated animals. In Experiment 1 with L1210/MTX, a 50% stimulation of deoxycytidine incorporation was observed after in vitro Methotrexate, and a 20% inhibition occurred in Experiment 2. In general, a correlation was observed between the effect of Methotrexate on deoxycytidine and thymidine utilization. A pronounced stimulation of the utilization of exogenous thymidine with inhibition of the de novo pathway of thymidine 5’-monophosphate synthesis was not observed.

DISCUSSION

Some similarities were noted in response to Methotrexate in the three transplantable ascitic leukemias with varying sensitivity to Methotrexate. For example, in all three lines, dihydrofolate reductase was inhibited within five minutes after the in vivo administration of the therapeutically optimal dose of Methotrexate for L1210. The difference in the rate of transport of Methotrexate reported earlier did not result in a failure to inhibit the enzyme even though L1210/MTX, the line with drug-induced resistance, had 5 times the level of enzyme activity observed in the parent line and 10 times the level observed in P329, an innately insensitive cell line. The rate of recovery of the enzyme activity in L1210/MTX after administration of 1.5 mg of Methotrexate/kg resembled the recovery noted in L1210 after a nontherapeutically effective dose, 0.2 mg/kg. Hakala (11) and Fischer (8) have suggested that drug-induced resistance in cell cultures of mouse neoplasms may be related to an increased rate of enzyme synthesis which exceeds the rate of drug uptake. If this were the case, then an increased rate of enzyme synthesis coupled with a reduced rate of drug uptake would allow a resistant cell to avoid drug intoxication by replacing the inhibited enzyme more rapidly as the concentration of drug fell in the host. Since the drug was rapidly cleared from the peritoneal cavity, this would suggest an increased rate of enzyme recovery as the basis of resistance for L1210/MTX until other data are taken into consideration.

If the action of Methotrexate is to kill the cell by blocking dihydrofolate reductase, the present data suggest that cells may

D. Roberts, unpublished observation.
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be able to survive for approximately four hours in the absence of the enzyme. This is the period which elapses before free enzyme begins to reappear in L1210/MTX after the administration of 1.5 mg of Methotrexate/kg. If the growth of the cells were synchronized by the first dose of drug, this period might be increased if the administration of subsequent doses of Methotrexate coincided with the G1 or G2 phase.

If the inhibition of dihydrofolate reductase leads to an oxidation of the "tetrahydrofolate" pool, then the incorporation of deoxyuridine into DNA might be increased if the administration of subsequent doses of Methotrexate is delayed by four hours. The capacity for the in vitro incorporation of deoxyuridine into DNA was only observed at the highest dose of Methotrexate, 150 mg/kg. A differential inhibition in vivo of thymidine 5'-monophosphate synthesis relative to purine synthesis may have occurred at this high dose but was not observed in vitro by a differential inhibition of thymidine incorporation when compared with deoxyxycytidine utilization (Chart 9). A statistically significant inhibition of deoxyuridine incorporation into DNA was observed. No statistically significant change was observed after the administration of Methotrexate in the incorporation of either thymidine or deoxyuridine into DNA by the two other cell lines.

The cells were repeatedly washed in the process of isolation for in vitro studies. If the inhibition of deoxyuridine incorporation observed in vivo were dependent upon an excess of free drug, i.e., drug in excess of the amount required for the "stoichiometric" inhibition of dihydrofolate reductase, then the poor response observed in vitro after the in vivo administration of Methotrexate could result from an efflux of the drug during isolation of the cells. The in vitro incubation of ascites cells with Methotrexate resulted in an inhibition of the incorporation of deoxyuridine into DNA by L1210 and P329 which would support this postulation that some drug was washed out of the cells during their isolation. The inhibition by Methotrexate had first order kinetics. L1210/MTX was insensitive to Methotrexate at 3.5 x 10^-5 M.

By the in vitro assay, dihydrofolate reductase activity was inhibited in all three cell lines by in vivo Methotrexate administration. If we assume that the same effective inhibition of dihydrofolate reductase existed in the intact cell in vitro, it may be necessary to postulate a second action by Methotrexate. This could be another site of inhibition or, possibly, a displacement of tetrahydrofolate derivatives from the cells. But, if the inhibition of dihydrofolate reductase is assumed to be the only site effectively inhibited by Methotrexate, there is the necessity of explaining why only stoichiometric amounts of drug equivalent to the level of dihydrofolate reductase are retained in liver and duodenal mucosa (23).

The present studies suggest that the requirement for "free" drug is the pharmacologic basis for the report that high levels of Methotrexate are effective in treating human acute leukemia which is resistant to lower daily doses (6). A lack of correlation was observed between the inhibition of dihydrofolate reductase activity in white cells from the human with leukemia and the response of the patient to Methotrexate (18, 19). The basis of the relationship between the rate of drug uptake and the prolongation of the lives of mice bearing transplantable ascites tumor cells apparently results from the necessity of maintaining levels of drug in excess of the amount required for the stoichiometric inhibition of dihydrofolate reductase.

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