Purineless Death as a Link between Growth Rate and Cytotoxicity by Methotrexate

William M. Hryniuk

Department of Medicine, University of Manitoba, and The Manitoba Institute of Cell Biology, 700 Bannatyne Avenue, Winnipeg, Manitoba R3E 0V9, Canada

SUMMARY

L5178Y murine lymphoblasts from cultures proliferating at different rates were treated with methotrexate (MTX). DNA, RNA, and protein synthesis and cell viability (cloning efficiency) were assessed during treatment. MTX suppressed incorporation of thymidine-3H into DNA, of uridine-3H into RNA, of leucine-3H into protein, and killed cells. The greater the proliferative rate, the greater were the drug effects. In fact, the cell kill could be predicted from the suppression of thymidine-3H incorporation. However, the cell kill did not cause the suppression. Hypoxanthine completely prevented these biochemical effects of MTX and partially protected the cells against its lethal effects. Thus, in the murine S-phase lymphoblasts, MTX produces a purineless state, the degree of which depends upon the growth rate of the population. The purineless state probably contributes to the lethal effect of MTX, and this may partly explain the relation between growth rate and cytotoxicity by antifolates.

INTRODUCTION

The faster cells proliferate, the more are killed by antimitabolites (24). Antimitabolites may kill cells from rapidly proliferating populations more efficiently because a greater fraction of the total cell population traverses the drug-sensitive phase of the cycle during treatment and, therefore, a greater fraction becomes a target for the drug (20, 24). The target cells, i.e., those reaching the drug-sensitive phase, should then have the same probability of being killed by the drug regardless of the growth rate of their parent population. Then the biochemical disturbances upon which the lethal effects of the drug depend should be the same regardless of whether the target cells are from a rapidly or slowly proliferating population.

MTX, clinically a very useful antimitabolite (10), kills L5178Y murine lymphoblasts from log cultures much more rapidly than cells from resting cultures (13). However, the biochemical disturbances produced in these cells by MTX treatment depend very much on whether they are treated in a rapidly (log) or a slowly proliferating (resting) culture. For example, MTX suppresses TdR incorporation into DNA to a much greater degree in the S-phase cells of the log culture. It was suggested that this suppression is due to interference with de novo synthesis of purines (13).

The present study defines in quantitative terms the relationship between a biochemical disturbance (suppression of TdR incorporation) produced by drug treatment and the lethal effect (loss of cloning efficiency) that results. In addition, the mechanism whereby MTX suppresses TdR incorporation is investigated and found, indeed, to be due to interference with de novo synthesis of purines.

MATERIALS AND METHODS

Media and Chemicals. MTX (sodium salt) was a gift from Cyanamid of Canada, Montreal, Quebec, Canada; Fischer's medium and horse serum were obtained from Grand Island Biological Company, Grand Island, N. Y. Noble agar was obtained from Difco Laboratories, Detroit, Mich.; L-serine and hypoxanthine were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio; TdR-3H (specific activity, 1.9 Ci/mmmole), uridine-5-3H (specific activity, 1 Ci/mmmole), and leucine-4,5-3H (specific activity, 6 Ci/mmmole) were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y.; and Hyamine hydroxide was obtained from Nuclear-Chicago Corporation, Des Plaines, Ill. Scintillation fluid was prepared by standard methods (3).

Cells and Culture Techniques. Stock cultures of L5178Y mouse lymphoblasts with a doubling time of 10 hr in Fischer's medium and 10% horse serum (7) were subcultured. Cells were harvested at intervals from the subcultures while growing at different rates, and treated with MTX by various dose-time schedules. They were then washed free of drug and pulsed with tritiated precursors to measure the rate of precursor incorporation into macromolecules or cloned into soft agar to measure cell kill (4).

Rate Determinations. Previously published methods (13) were used for incorporation of TdR-3H. Uridine-3H was incorporated by the same methods as TdR-3H except that uridine-3H, 2 µCi/ml, was added to each flask. The cells were precipitated with trichloroacetic acid to stop the pulse labeling and were washed with trichloroacetic acid and ethanol. The uridine incorporated into RNA was extracted by hydrolyzing for 60 min at 37° in 0.5 N NaOH.

Leucine-3H was also incorporated by the same methods as TdR-3H, except that leucine-3H, 5 µCi/ml, was added to each flask. The acid-washed precipitates were dissolved in methanol-Hyamine and added to scintillation fluid.
Measurement of Radioactivity and Calculations.

Measurement of tritium, corrections for efficiency of counting, and calculation of rate constants, errors, and correlation coefficients were performed as previously described (13, 22). Rates were expressed as pmoles of tritiated precursor incorporated into the macromolecule per min of incubation per million cells. Rates in the presence of MTX were expressed as decimal fractions of the control rate and designated as "fractional rates."

RESULTS

Relationship between Suppression of TdR-3H Incorporation and Kill by MTX

Selection of Treatment Conditions. The conditions under which MTX suppressed TdR-3H incorporation were first delineated. Cells from early log cultures were harvested and treated with MTX over a range of concentrations and times. At each concentration the rate of TdR-3H incorporation was measured at intervals during 16 hr of treatment and the fractional rate was determined; Chart 1 depicts the results. No effects were noted at 1 x 10^-9 M MTX; suppression began at 10^-8 M and was maximum at 10^-6 M. Higher concentrations did not produce further suppression. The maximum suppression occurred at 4.5 hr at the higher doses, and in all experiments the rate of TdR-3H incorporation then rose despite continued MTX treatment. The same experiment was repeated with cells from resting cultures. In most studies with resting cultures, minimal effects on TdR-3H incorporation were noted at the highest doses of MTX tested for the 1st 8 hr of treatment. The conditions chosen for all further experiments were the minimum dose and time that produced the maximum suppression of TdR-3H incorporation into DNA in log culture cells, i.e., 1 x 10^-6 M for 4.5 hr.

Cultures of different growth rates were treated with these conditions and the effects of TdR-3H incorporation and cloning efficiency were measured. Suppression of incorporation was expressed as logs of decrease from the control rate. Cell kill was measured as logs of decrease from the control cloning efficiency. As shown in Chart 2, the rate of control TdR-3H varied from 3 pmoles/min/10^6 cells (log cultures) to 0.45 pmole/min/10^6 cells (resting cultures). The higher the rate of control TdR-3H was, the greater was the suppression produced by MTX (Chart 3). The relationship appears linear and is highly significant. Note that at the lower rate of control TdR-3H of resting culture cells MTX usually did not suppress incorporation. The greater the suppression the greater the kill (Chart 4). Again the relationship appears linear and the coefficient of correlation is highly significant.

Mechanism of Suppression by MTX of TdR-3H Incorporation

As suggested earlier (13), the suppression of TdR-3H incorporation pointed to a block in DNA synthesis, most
Chart 3. Control TdR-3H rate correlated with the degree of MTX suppression of that rate. Fractional TdR-3H rates were measured after 4.5 hr of exposure to 10^{-6} M MTX (see legend to Chart 1) and the logs of decrease from a fractional rate of 1.0 were plotted against the rate in the untreated cultures (control rate). Control rate = pmoles/min/10^6 cells on a linear scale.

Chart 4. Correlation between biochemical and cytocidal effect of MTX. The biochemical effect was calculated as in Chart 3. Kill was measured as a reduction in cloning efficiency.

likely due to interference with de novo synthesis of purines, in addition to the block of conversion of deoxyuridylate to thymidylate. If this suggestion is correct, then adding an exogenous source of purines should prevent this effect of MTX.

Cultures of different growth rates were treated with MTX in the presence and absence of 2 \times 10^{-5} M hypoxanthine and the effect of this combined treatment on TdR-3H incorporation into DNA was determined. Hypoxanthine completely prevented the suppressive effect of MTX on TdR-3H incorporation at all proliferative rates tested. Hypoxanthine by itself appeared to have little effect on TdR-3H incorporation (Chart 5).

Effect of MTX on RNA and Protein Synthesis

Since MTX appeared to be blocking de novo purine synthesis, the effect of this block on RNA and protein synthesis was next studied.

Rate of Uridine-3H Incorporation into RNA. Table 1 indicates that MTX suppressed uridine-3H incorporation in both log and resting culture cells, although there was 23-fold greater suppression in the log culture cells considering the differences in the control rates, and that hypoxanthine completely prevented these effects of MTX and, in fact, stimulated uridine-3H incorporation in the cells whether or not they were treated with MTX.

Rate of Leucine-3H Incorporation into Protein. MTX suppressed leucine-3H incorporation to a 2.5-fold greater degree in the cells from log cultures (compare a fractional rate of 0.42 versus 0.95 in Chart 6). This effect was prevented by hypoxanthine (Chart 7) but not by 1 \times 10^{-3} M serine.

Chart 5. MTX (1 \times 10^{-6} M) effects on TdR-3H rate in absence and presence of hypoxanthine (2 \times 10^{-5} M). Treatment was for 4.5 hr. TdR-3H rate measurement and units as in legend to Chart 2. The experiment was repeated with identical results. o, no additions; o, hypoxanthine alone; •, MTX alone; •, hypoxanthine and MTX.
Growth Rate and Cytotoxicity

Table 1
Effect of MTX on rate of uridine-$^3$H incorporation in presence and absence of hypoxanthine

Experimental design as in Chart 5, except that uridine-$^3$H incorporation into RNA was measured.

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Logarithmic culture</th>
<th>Resting culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.03 ± 0.14</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.021 ± 0.001</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Control + hypoxanthine</td>
<td>1.72 ± 0.05</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Methotrexate + hypoxanthine</td>
<td>2.45 ± 0.22</td>
<td>0.52 ± 0.04</td>
</tr>
</tbody>
</table>

* Averages from 2 separate experiments which did not vary from each other significantly.

Chart 6. MTX suppression of leucine-$^3$H incorporation into protein. Cells from log (early and late) and resting cultures were incubated for 4.5 hr with (•) and without (o) antifolate ($1 \times 10^{-6}$ M). The rate of leucine-$^3$H incorporation was then measured during a 20-min pulse as pmoles of leucine incorporated into protein/min of incubation/10$^6$ cells. The fractional rate of leucine-$^3$H was the rate in the presence of MTX divided by the control rate. The (geometric) means ± S.E. are from 3 experiments.

Hypoxanthine Protection against Lethal Effects of MTX

Hypoxanthine partially prevented the cytotoxic effects of MTX when purine and antifolate were added to the cultures simultaneously. The protection increased as the concentration of hypoxanthine was raised from $2 \times 10^{-7}$ M to $2 \times 10^{-5}$ M. It did not potentiate the lethal effect of MTX at any concentration. The optimum concentration of hypoxanthine chosen for subsequent studies was $2 \times 10^{-5}$ M. This gave partial protection which was most noticeable in early log cultures but was present even in resting cultures (Chart 8). Adding hypoxanthine after MTX treatment did not rescue the cells.

Chart 7. MTX ($1 \times 10^{-6}$ M) effects on leucine-$^3$H incorporation into protein. Treatment was for 4.5 hr in the presence (c, o) and absence (e, ●) of hypoxanthine. See legend to Chart 6 for measurement of the fractional rate of leucine-$^3$H. The experiment was done twice (● o; ● o).

Chart 8. Effect of hypoxanthine on kill of cells by MTX. Cells from cultures of different growth rates were treated with MTX (4.5 hr, $1 \times 10^{-6}$ M) in the absence and presence of hypoxanthine ($2 \times 10^{-5}$ M), then washed and cloned. The experiment was repeated with identical results. o, no additions; o, hypoxanthine alone; ●, MTX alone; ●, hypoxanthine and MTX.
DISCUSSION

Incorporation of TdR-3H was greatest in cells from the most rapidly proliferating cultures, probably due only in part to a higher labeling index in log (62%) compared to resting (50%) cultures (26).

The higher the rate of control TdR-3H was, i.e., the faster the growth rate, the greater was the suppression produced by MTX. The greater the suppression, the greater the kill. The reduction in clonning efficiency (cell kill) could be predicted from the biochemical effects on the S-phase cells. Thus, the proliferative rate may govern susceptibility to MTX by making the S-phase cells more sensitive to lethal biochemical disturbances produced by the antifolate.

In vitro measurement of the effects of MTX on TdR-3H incorporation by human acute leukemia cells may likewise predict the leukemocidal effects of MTX and, to a certain extent, the outcome of antifolate therapy. Such an in vitro system might partially substitute for sorely needed clonning systems for human tumors. This would be an invaluable guide to chemotherapy and might explain why most tumors resist MTX therapy. Of course, such a system would not assess G0 cells.

In the L5178Y lymphoblasts, the suppression of TdR-3H incorporation is probably not due to kill. Initially, suppression is faster than kill (13) but then lifts while kill continues (cf. Fig. 3A in Ref. 13 and Chart 1). Further, a small degree of kill may occur in the resting cultures without suppression of TdR-3H incorporation. Hypoxanthine completely corrects the suppression but only partially prevents the kill. The reason for the lifting of suppression despite continuing kill is unknown but it is not due to increased activity of TdR kinase (unpublished data).

MTX suppresses incorporation of TdR-3H by impairing de novo purine synthesis since hypoxanthine prevents this effect. Presumably, reduced folates, required in purine ring construction, are depleted when deoxyxuridylate converts to thymidylate (25, 27) in the presence of MTX. MTX may also directly interfere with enzymes concerned with purine ring construction (2). Lack of purines does not decrease TdR kinase, as occurs in other systems (Ref. 6; unpublished data).

In L5178Y cells, MTX-induced lack of purines also blocks RNA synthesis and probably protein synthesis since hypoxanthine prevents the suppression of uridine-3H into RNA and of leucine-3H into protein. On the other hand, serine does not prevent MTX-induced suppression of leucine-3H incorporation into protein. Thus, the block of protein synthesis may not be due to inhibition of glycine conversion to serine (27) or interference with histidine synthesis (23) as has previously been assumed.

The block by MTX of de novo purine synthesis has been assigned a minor role in cell killing (14). In fact, under certain conditions (1, 16), this block protects cells from MTX, while added purines accentuate the effects of the antifolate. However, as shown in the present study, L5178Y cells die a purineless death as well as a thymineless death, at least during the first 4.5 hr of exposure to antifolate, because added purine partially prevents kill. Similarly, the data from studies in humans (11, 15), animals (8, 18, 19, 21), and other culture systems (9) suggest that the purineless state induced by MTX may add to the cytocidal effects of the antifolate.

The purineless state, by impairing RNA synthesis, may impair synthesis of new enzymes. In effect, then, by inhibiting activity of the reductase, MTX would impair synthesis of new reductase and the cell would be unable to overcome the block even if extracellular MTX were removed. This might have some relevance to the clinical situation. In acute leukemia cells harvested from humans during MTX therapy, dihydrofolate reductase protein accumulates during therapy in some cases while in others it does not. The best responses to therapy, i.e., the greatest kill of leukemic cells, occur when enzyme protein does not accumulate (11, 28).

Allopurinol inhibits xanthine oxidase and, when given to patients receiving chemotherapy for malignant diseases (17), may cause accumulation of hypoxanthine. This might complicate the cytocidal action of MTX, but the effects would be difficult to predict since hypoxanthine might protect some cells and hasten the death of others.

The current studies suggest that kinetics of proliferation govern cell kill indirectly by modulating the metabolic disturbances induced in the S-phase cells. In other cell types the effects of MTX on S-phase cells also shift with the proliferative rate (12). Two types of cells may proliferate at the same rate, yet the lethal effects of MTX might be different because different biochemical disturbances may be produced in the S-phase cells of the 2 populations (12); i.e., nonkinetic parameters may determine what the effect of MTX on S-phase cells will be, and, therefore, what cytotoxic effect will result from treatment. Such superimposed nonkinetic influences may make some malignant cells more sensitive to antimetabolites than normal cells despite the fact that malignant cells may be proliferating more slowly than normal cells (5).

ACKNOWLEDGMENTS

The technical assistance of Mrs. Diane Johnson and the helpful discussions and constructive criticisms of Dr. J. Borsa are gratefully acknowledged.

REFERENCES

Purineless Death as a Link between Growth Rate and Cytotoxicity by Methotrexate

William M. Hryniuk


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/32/7/1506

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.