The Effect of Methotrexate on Transformation and Mitosis of Normal Human Blood Lymphocytes In Vitro

By L.A. Rozenszajn and J. Radnay

The effect of methotrexate (MTX) on transformation and mitosis of normal lymphocyte cultures was investigated. Mitosis was 1000-fold more sensitive to MTX than was transformation; mitosis could be prevented without any observable effect on transformation. The antimitotic effect of MTX depended on its concentration, duration of contact with lymphocytes, as well as on the developmental stage of the lymphocytes at the time of exposure. Incorporation of labeled precursors into protein, DNA, and RNA proceeded at normal rates unaffected by the presence of MTX. Transformation was partially inhibited by high concentrations of MTX (above 75 µg/ml). Folinic acid abolished the antimitotic effect of MTX, with the number of cells undergoing division directly proportional to the quantity of folinic acid added.

The use of methotrexate (MTX, 4-amino, 10-pteroyl glutamic acid) in cancer chemotherapy was based on its strong inhibition of dihydrofolate dehydrogenase (7.8 dihydrofolate: NADP oxidoreductase 1.5.1.4)1,2 and the resulting failure to produce tetrahydrofolate acid (THFA). This inhibition interferes with transfer of one-carbon units, which, in turn, affects synthesis of purine and pyrimidine, as well as that of methionine, serine, and glycine. The interference with nucleic acid synthesis affects rapidly dividing tumor cells much more than normal body cells.3

Most of this work was done on cells of leukemic patients in whom a correlation was found to exist between activity of dihydrofolate dehydrogenase and response to MTX.4,5

The inhibitory effect of MTX on dihydrofolate dehydrogenase has been examined in various biological systems.6,10,4,5 The chemical reaction between the enzyme and MTX has been investigated as well.11,3 On the other hand, folinic acid had the property of canceling the inhibitory effect produced by MTX.12

Since nonspecific stimulation, such as addition of phytohemagglutinin (PHA), could transform cultured lymphocytes into blast-like forms and induce mitosis, we decided to study the effect of MTX on these processes in cultures of normal human blood lymphocytes. This system enabled us also to study the effect of MTX at various stages of cell development.
MATERIALS AND METHODS

Culture Method

Human lymphocytes, taken from eight healthy volunteers, were cultured by the method of Moorhead with slight modifications, using PHA to induce transformation and mitosis of the lymphocytes. Venous blood samples (between 20 and 200 ml) were taken by means of disposable plastic syringes containing 10 IU/ml heparin (pyrogen-free, Evans). The lymphocytes were isolated from the other white blood cells on glass columns according to the method of Rabinowitz.

Each tissue culture tube contained $8 \times 10^6$ lymphocytes, 2 ml autologous plasma, 0.1 ml Bactophytohemagglutinin M (Difco), 0.2 ml MTX (Methotrexate sodium, Lederle) in different concentrations, medium TC 199 (Difco) up to 8 ml. The medium contained 1000 IU/ml penicillin and 2 mg/ml streptomycin. In the experiments with labeled thymidine, lymphocytes were also cultured in Eagle medium. The control cultures did not contain MTX. In some experiments, folic and folinic acid (K & K laboratories), $^3$H-thymidine, $^{14}$C-phenylalanine, or $^3$H-uridine (Amersham, Radiochemical Center), were added to the blood-cell cultures. Duplicate tubes of lymphocyte cultures were set up in each experiment.

Estimation of Lymphocyte Transformation and Mitotic Index

The morphology of the cultured cells was examined in smears stained according to May-Grünwald Giemsa, after 72 hr of culture, and the percentage of transformed lymphocytes, i.e., large cells with basophilic cytoplasm, was determined by counting 1000 cells in each smear.

After 72 hr of culture, Colcemid (20 μg/ml) was added, and 3 hr later the mitotic index (number of mitosis per 1000 blasts) was estimated, and chromosome preparations were made. By comparing numbers of transformed lymphocytes and the mitotic index in MTX-treated cultures with corresponding values in control cultures, the effect of MTX was expressed as "relative lymphocyte transformation" and "relative mitotic index."

Effect of Incubation Time With MTX (50 μg/ml) on Lymphocyte Cultures

After different intervals of time, 30 min, 1, 2, 3, 18, and 24 hr, the antimetabolite was removed by centrifugation and washing of the cells three times with medium TC 199. The cells were re-suspended with all culture components, except for MTX. The mitotic indices were checked after 72 hr of incubation. In order to determine the antimitotic effect in different stages of culture development, MTX was added to lymphocyte cultures after 0, 24, 48 hr from the beginning of the culture, and after incubation for 72 hr, the mitotic indices were estimated.

Chromosome Studies

Karyotypes were analyzed using phase-contrast microscopy; representative cells were photographed on 35 mm Microfile Kodak or AGPFF Agfa, and karyotypes were prepared from the enlarged prints. The Denver system of nomenclature was used (Denver Study Group, 1960).

Study of Protein, RNA, and DNA Synthesis

Protein, RNA, and DNA synthesis by cultured lymphocytes was studied with and without the addition of MTX (50 μg/ml) by measuring incorporation into the cells of $^{14}$C-phenylalanine, $^3$H-uridine, and $^3$H-thymidine. The radioactive agents were added at the beginning of the culture in a final concentration of 0.1 μCi/ml. In experiments in which Eagle medium was used, the radioactive agents were added 20 hr after initiating the cultures. Three parallel lymphocyte cultures were prepared in each experiment: one with PHA and MTX, one with PHA only, and one control culture with neither of them.

Samples of 1 ml were taken at the beginning of the culture and after 24, 48, and 72 hr of incubation. The samples were centrifuged, the cells were washed in phosphate buffer saline (pH 7.4), and subjected to freezing and thawing in dry ice three times. The macromolecules of the lysed cells were precipitated in $10%$ cold trichloroacetic acid. The suspension was filtered through a millipore filter (Millipore Filter Corp.). The filters were dried at 80°C for 1 hr. Each filter was transferred to liquid-scintillation vials containing 10 ml scintillation fluid (toluene with POPOP.
and PPO) and measured in a liquid-scintillation counter (Packard "Tri-Carb" Scintillator) for 10 min.

**Extraction and Quantitative Estimation of Nucleic Acids**
RNA and DNA were extracted from cultured lymphocytes according to the method of Feinendegen et al., as modified by Cooper and Rubin. The optical densities of the DNA and RNA extracts were measured in a spectrophotometer at wavelengths of 268 and 260 nm, respectively. The standards were prepared from purified DNA and RNA (Sigma).

**Abolition of the Effect of MTX (50 μg/ml) on Lymphocyte Cultures**
MTX and either folic acid or folinic acid were added to cultures, the latter in concentrations ranging from that of MTX to eightfold concentration. The mitotic indices were checked after 72 hr. In additional experiments, folinic acid was added to cultures after 18 hr of incubation.

**Influence of Lymphocyte Lysates on Lymphocytes Cultured in Presence of MTX**
Lymphocyte cultures were prepared in the presence of MTX. The MTX was removed from the culture after 18 hr of incubation by washing of the cells three times with medium TC199. Lysates of normal cultured lymphocytes were added to the washed cells, and medium TC199 was added to each culture up to the volume of 8 ml.

The lysates were prepared from lymphocytes cultured for 24, 48, or 72 hr; the cells were washed three times in medium TC 199, and lysed by freezing in a mixture of aceton and dry ice and subsequent thawing.

**RESULTS**
At MTX concentrations below 50 μg/ml, the transformation of lymphocytes was not affected, and 80%-90% of the cells were transformed into large pyroninophilic and blast-like cells. When the MTX concentration was 75 μg/ml or higher, the percentage of transformed cells dropped to 50% and remained unchanged, with MTX concentrations up to 400 μg/ml (Fig. 1). The relative mitotic index dropped to 50% when the MTX concentration was 0.1 μg/ml, and at 0.35 μg/ml no dividing cells could be found in the culture (Fig. 2). The inhibition of mitosis was complete 3 hr after initiation of the culture. Contact of 1 hr between MTX and the cells decreased the relative mitotic index to 25% (Fig. 3). The inhibition of mitosis was complete only in those experiments in which the MTX was added for the first 24 hr of culture. Addition of MTX 48 hr after initiation of the culture reduced the mitosis to 90% as compared with control cultures. Mitosis appeared in all lymphocyte cultures with low MTX concentrations (below 0.1 μg/ml). The karyotypes of these mitoses were perfectly normal.

![Fig. 1. Effect of MTX on transformation of lymphocytes in culture.](image-url)
Study of Protein, RNA, and DNA Synthesis

There was no difference in the uptake of $^3$H-phenylalanine between the lymphocyte cultures with and without MTX. In the control culture without PHA, the uptake of $^3$H-phenylalanine was very low (Fig. 4). The uptake of $^3$H-uridine by lymphocytes cultured with and without MTX was similar. In the control culture without PHA, the uptake of $^3$H-uridine was very low (Fig. 5). The uptake of $^3$H-thymidine by lymphocytes in culture was not affected by the presence of MTX. In the control culture without PHA, the uptake of $^3$H-thymidine was very low (Fig. 6). In cultures containing Eagle medium, the uptake of $^3$H-thymidine by lymphocytes cultured with MTX was higher than that of lymphocytes cultured without MTX (Fig. 7). The ratio DNA/RNA was somewhat higher in lymphocyte cultures with than without MTX (Table 1).

Abolition of the Effect of MTX in Lymphocyte Cultures

The mitotic inhibition of MTX was completely nullified by addition of folic acid at the beginning of the culture in a concentration which was four times that of MTX (Fig. 8). When folic acid was added 18 hr after the beginning of the culture, mitosis appeared 18 hr later. Folic acid did not affect the inhibition of mitosis, even in concentrations eight times that of MTX. Addition of lysates
Fig. 4. $^{14}$C-phenylalanine incorporation in presence of MTX (50 µg/ml) and/or PHA in lymphocyte culture. control; + PHA; + PHA + MTX.

Fig. 5. $^3$H-uridine incorporation in presence of MTX (50 µg/ml) and/or PHA in lymphocyte culture. control; + PHA; + PHA + MTX.

Fig. 6. $^3$H-thymidine incorporation in presence of MTX (50 µg/ml) and/or PHA in lymphocyte culture. control; + PHA; + PHA + MTX.
Fig. 7. $^3$H-thymidine incorporation in presence of MTX (50 μg/ml) and/or PHA in lymphocyte culture (Eagle medium). 

Table 1. Nucleic Acid Content of Lymphocytes Cultured in the Presence or Absence of MTX

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Lymphocyte Culture Without MTX</th>
<th>Lymphocyte Culture With 50 μg/ml MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>1</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*μg/10⁶ cells.

Fig. 8. Abolition of inhibition of mitosis by folic acid. Case 1; Case 2.

M - 50μg/ml MTX
F - 50μg/ml FOLINIC ACID
prepared from cultured lymphocytes had no effect on the inhibition of mitosis by MTX.

DISCUSSION

Using human lymphocytes cultured in presence of MTX, we investigated the influence of this antimetabolite on transformation of the lymphocytes to blast-like cells and on PHA-induced mitoses. Despite the appearance of blast-like cells after 72 hr culture, the mitotic activity was completely abolished.

From the above experiments, it was clear that mitosis of cells in culture was 1000-fold more sensitive to MTX than is transformation and that mitosis could be completely prevented without any observable effect on transformation of lymphocytes to blast-like cells. The influence of MTX on cell division depended on its concentration, the duration of contact with the cells, and the mitotic phase during which the cell is exposed to the antimetabolite.

A cellular factor seems to be involved, since removal of the MTX from the culture after 3 hr of contact did not abolish the inhibitory effect on cell division (Fig. 3). The addition of MTX to a 48-hr culture, on the other hand, did not impair the division of a part of the cell population. Thus, it may be assumed that these cells were in a phase of the mitotic cycle which is not affected by the antimetabolite.3 On the other hand, the incorporation of labeled precursors into DNA, RNA, and protein was unaffected by presence of MTX.

It has been stated that MIX caused THFA deficiency in biologic systems1,2 and that the most sensitive stage of this deficiency concerns the formation of deoxyuridylate (dUMP) from thymidylate (dTMP).7

It is well established that transformation is accompanied by synthesis of nucleic acids and proteins.18,21 Since MTX interfered with one-carbon chain metabolism, it is therefore expected to inhibit this synthesis. It was of interest to study the effects of MTX on nucleic acid and protein synthesis, in a concentration which completely inhibited mitosis (50 µg/ml), in culture medium containing amino acids, thymine, and deoxyribose, utilized by the cell in thymidine synthesis (e.g., TC 199), and in culture medium not containing purines and pyrimidines, such as Eagle medium.

In a pyrimidine- and purine-free medium (Eagle medium) the incorporation of thymidine was much higher in the presence of MTX than in its absence (Fig. 7). A possible explanation is that cellular synthesis of dTMP was inhibited by MTX. Therefore, the labeled thymidine in the medium was the sole source available for DNA synthesis.

These results contrast with those obtained when medium T.C. 199 was used (Fig. 6) and with the control where labeled thymidine was diluted with the internal dTMP pool maintained by the cell (Fig. 7). A possible explanation for the lack of influence of MTX on thymidine incorporation (Fig. 6) was availability of an alternative pathway through thymidine kinase. In the presence of this enzyme, the thymidine could convert to dTMP, and thus the need for THFA is avoided. It might be that, due to the blocking of the main dUMP-dTMP pathway, the synthesis through the alternative kinase pathway was accelerated.17,24,26 The influence of MTX on thymidylate synthetase activity varied with the systems investigated.26,29
The results of the experiments in the two different tissue-culture media support the suggestion that when the main pathway of dTMP was blocked, the alternative pathway was used. Although DNA synthesis was not inhibited by MTX, the newly synthesized nucleic acids may be structurally abnormal, causing inhibition or prevention of mitosis. Our chromosomal studies did not reveal chromosome breakage, which was reported to occur in plants and patients treated with MTX. In those of our experiments in which concentration of MTX did not prevent mitosis, the chromosomes were normal.

The presence of the antimetabolite in the culture had no effect on phenylalanine incorporation into proteins (Fig. 4), although cell division was completely inhibited at an MTX concentration of 0.17 μg/ml. However, deficient synthesis of a particular, essential protein cannot be excluded. The fact that folic acid did not neutralize the antimimetic influence of MTX, whereas folinic acid did repair this damage, indicates that mitotic inhibition is connected with THFA deficiency.

In these experiments, we found that the inhibition could be partially overcome by the presence of equal amounts of folic acid, in the presence of which the mitotic index reached 25% of the control (Fig. 8), whereas other lymphocytes required a larger quantity of folic acid in order to undergo cell division. The reason for this phenomenon may be that the culture was not synchronic.

A 3-hr contact between cells and MTX is enough to depress the mitotic process (Fig. 3). This inhibition can be reversed by the addition of folic acid not later than 18 hr after initiation of the culture. Under these conditions, mitosis will appear with a delay of 18 hr, as compared with control cultures without MTX. This retardation indicates that the process of lymphocyte transformation to blast-like cells is not necessarily followed by cell division, and that the effect of MTX is mediated by its inhibition of dihydrofolic acid dehydrogenase and not by side effects.

Another possibility is that THFA has some additional function, besides synthesis of nucleotides and proteins, and that this function is directly related to mitosis. We failed to induce mitosis in cultures in the presence of MTX by adding proteins prepared as lysates from cultured lymphocytes. Mitotic activity of such proteins was therefore not proven. The fact that mitosis is delayed in cultures with MTX to which folic acid is added later led us to additional experiments on long-term lymphocyte cultures treated with MTX, in order to study the possibility of the synthesis of new THF in these cells, which should be expressed by renewal of cell division. Preliminary experiments tend to confirm this hypothesis.

REFERENCES
5. Canallos GP, Mead JAR, Greenberg NH,
15. Rabinowitz Y: Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. Blood 23:811, 1964
32. Taylor JH: DNA synthesis in relation to chromosome reproduction and reunion of breaks. J Cell Comp Physiol (suppl) 1:73, 1963
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