Mechanism of Growth Inhibition by Methotrexate

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SUMMARY

The effect of methotrexate on the growth of Chinese hamster cells was examined under a variety of conditions. The experiments suggest that the important biological effects of methotrexate are the result of direct inhibition of thymidylate synthetase and one or both of the folate-dependent enzymes involved in purine biosynthesis. In addition, analysis of the distribution of intracellular folate derivatives following methotrexate treatment gives no indication of accumulation of dihydrofolate, an accumulation that would be expected if inhibition of dihydrofolate reductase were the principal site of methotrexate action.

INTRODUCTION

It has been well established that MTX (amethopterin) is a potent inhibitor of the enzyme dihydrofolate reductase (EC 1.5.1.3) (1). This enzyme has 2 functions in vivo: to reduce the vitamin FA to its biologically active form of FH$_4$ and to reduce FH$_2$ to FH$_4$. FH$_2$ being the folate cofactor product of the reaction catalyzed by thymidylate synthetase (EC 2.1.1.2).

Although it has been shown that MTX inhibits thymidylate synthetase (3, 6), it is often assumed that the inhibition of FH$_4$ reductase in vivo is responsible for all of the biological effects of MTX that include inhibition of the synthesis of thymidylate, purines, and glycine (1). The suggested mechanism by which this occurs is as follows. MTX is assumed to inhibit the conversion of FH$_4$ to FH$_4$ so that as thymidylate is synthesized by thymidylate synthetase, 5,10-methylene-FH$_4$ is converted to FH$_2$, which cannot be reduced and must therefore accumulate. A consequence of this accumulation of FH$_2$ is the depletion of the cellular pool of biologically active FH$_4$ and FH$_4$ derivatives, which are required for the synthesis of thymidylate, purines, and glycine.

This single-site-of-action hypothesis requires that all or most intracellular folate derivatives accumulate as FH$_2$ in MTX-treated cells. We tested this prediction directly by analyzing the distribution of folate derivatives in Chinese hamster cells cultured in vitro. In contrast to a large increase in FH$_4$ predicted both by the previously mentioned model and by a computer simulation of folate metabolism (8), we found no evidence for FH$_2$ accumulation in MTX-treated cells.

MATERIALS AND METHODS

Chemicals. The MTX used in these experiments was purchased from Lederle Products of Cyanamid of Canada, Montreal, Canada. FA labeled with tritium in positions 3', 5', and 9 was purchased from Amersham/Searle Corp., Arlington Heights, Ill.

Cell Lines. Experiments were performed with a Chinese hamster ovary (CHO) line (12) and with a mutant derivative AUXB3, which is auxotrophic for glycine and a purine source (9). Suspension cultures of both cell lines were maintained at 34°C in α medium (15) supplemented with 10% fetal calf serum.

Chromatography of Folates. Cellular folates were labeled by growing cells for 48 hr in the presence of $^3$H-labeled FA (1 μCi/ml, 30 Ci/m mole) in α medium lacking only FA. Cells were then centrifuged and resuspended in α medium which either did or did not contain MTX (0.1 μg/ml). After 24 hr of incubation in this medium, the cells were washed with PBS; resuspended in a solution of 1% sodium ascorbate and 0.005 M phosphate buffer, pH 7.0; and placed in a 90° water bath for 20 min. The suspension was then centrifuged at 1000 rpm, and the supernatant which contained more than 85% of the radioactively labeled intracellular folates was applied to a DEAE-cellulose column (0.9 x 5 cm). Columns were eluted at a rate of about 1.0 ml/min with a linear gradient of 0.005 to 0.5 M sodium-potassium phosphate buffer, pH 7.0 (11), containing 1% β-mercaptoethanol. Approximately 50 fractions of 1 ml each were collected, and the radioactivity in each fraction was determined in a scintillation counter after the addition of 5.0 ml of Aquasol (Amersham/Searle Corp., Arlington Heights, Ill.) per fraction. The presence of MTX during the last 24 hr did not affect the total recovery of radioactive intracellular folates from either cell line. Virtually all (greater than 95%) of the radioactivity was recovered from the columns loaded with folates extracted from AUXB3.
About 5% of the radioactivity from wild-type extracts was not eluted from the column with the phosphate concentrations used.

**Growth Experiments and Media.** Growth experiments were carried out in suspension cultures carried in plastic tubes containing 10 ml of medium (2). Aliquots of 0.5 ml were removed daily, and the cell concentration was determined with an electronic cell counter. The -GAT medium used for some growth experiments consisted of α medium lacking glycine, all nucleosides, and all deoxynucleosides. The medium referred to as -G consists of -GAT medium supplemented with adenosine and thymidine. Similarly, -A and -T media were constructed from -GAT medium. These media all contained methionine (15 mg/liter) and were supplemented with 10% fetal calf serum which had been dialyzed against 1000 volumes of PBS (5). This dialysis procedure reduced folate activity in the serum [as determined by *Lactobacillus casei* (6)] from 15.5 ng/ml to 1.5 or 0.15 ng/ml in the growth medium. This value appears to be sufficiently low since CHO cells will not grow in -GAT medium containing 10 μg of folate per ml. To initiate cultures for growth experiments, cells growing in α medium were centrifuged, washed with PBS, and seeded into the appropriate media to form suspension cultures, which were incubated at 34°C.

**RESULTS**

**Chromatographic Analysis of Cellular Folates.** The 1st series of experiments were designed to determine whether intracellular folates accumulate as FH₄ in MTX-treated cells. In order to label intracellular folates, wild-type and AUXB3 cells were grown for 48 hr in the presence of ³H-labeled FA. The cells were then resuspended in medium containing MTX and incubated for an additional 24 hr. During this 24-hr exposure to MTX, cells were in α medium containing thymidine, adenosine, and glycine, and consequently growth was exponential and not inhibited by the MTX present. The concentration of MTX used, 0.1 μg/ml, was sufficiently high to inhibit cellular synthesis of thymidylate and purines (see Chart 2).

The ³H-labeled folates extracted from wild-type cells were analyzed by chromatography on DEAE-cellulose, and the results are shown in the left panels of Chart 1. The elution profile of folates from untreated cells was similar to those obtained for folates extracted from other animal tissues (11, 13), where the majority of activity appeared to be associated with folylpentaglutamates which eluted from the column at high phosphate concentrations (at high fraction numbers). The tentative identity of each peak is recorded in the chart legend. The total amount of intracellular folate recovered from MTX-treated cells was equal to that recovered from untreated cells. The chromatographic profile of the folates extracted from MTX-treated cells was similar to that of untreated cells, indicating that MTX altered neither the distribution of glutamyl residues conjugated to the folate derivatives nor the distribution of 1-carbon derivatives associated with the pentaglutamates.

Although most (85 to 90%) of the folates extracted from mammalian tissue appear to be pentaglutamates (7, 11), mono-, di-, and triglutamate forms also exist and it is possible that *in vivo* these forms are the important cofactor sources. The relevant FH₄ accumulation due to MTX might occur in these lower glutamate forms and may not be noticed by analysis of wild-type folates because of the excess of pentaglutamate forms. We therefore analyzed the folates extracted from AUXB3, a cell line that contains primarily mono- and diglutamate folate derivatives (9). As shown in the right panels of Chart 1, the elution profiles obtained from folates extracted from untreated and MTX-treated AUXB3 cells were similar. The distribution of folates was unaltered in AUXB3 cells grown in the presence of MTX and no detectable accumulation of FH₄ (which elutes from these columns in Fraction 30) occurred.

**Growth Inhibition Studies with MTX.** Since the above results suggest that the single-site-of-action model is incorrect, attempts were made to elucidate the mechanism(s) by which MTX inhibits the folate-dependent synthesis of thymidylate, purine, and glycine in wild-type cells. Cell growth assays were carried out in medium lacking only thymidine (-T medium), purines (-A medium), or glycine (-G medium). The growth inhibition produced by MTX resulted from inhibition of the synthesis of the nutrient...
omitted because cells could be maintained in exponential growth continuously in the presence of MTX if the medium contained thymidine, a purine such as adenosine, and glycine.

The effect of various concentrations of MTX on the growth of cells in -T, -A, and -G media is shown in Chart 2. At concentrations greater than 0.1 \( \mu g/ml \), MTX brought about immediate cessation of growth in -T and -A media; however, these same concentrations of MTX did not bring about inhibition in -G medium until a 5- to 7-fold increase in cell number had occurred. While all concentrations of MTX tested allowed a 5- to 7-fold increase in cell number in -G medium even at concentrations as low as 0.01 \( \mu g/ml \), there was pronounced inhibition beyond this point and the degree of inhibition appeared relatively unchanged over the concentration range from 0.01 to 1.0 \( \mu g/ml \). These results contrast sharply with those seen in -T and -A media where concentrations as low as 0.1 \( \mu g/ml \) produced marked inhibition almost immediately upon addition and where there was a much greater spread in the effects produced by lower drug concentrations. These results suggest that glycine synthesis was inhibited in MTX-treated cells by a mechanism different from that (those) responsible for inhibition of thymidylate and purine synthesis.

MTX has been found to inhibit the reduction of FA in vivo (4). To determine the significance of this inhibition and the concentration of MTX at which it occurs, the following experiment was carried out. Cells were cultivated for 1 week in medium containing thymidine, purines, and glycine but lacking FA in order to deplete the intracellular folate pool. These folate-depleted cells were then washed and resuspended in medium lacking thymidine, purines, or glycine and containing FA (1 \( \mu g/ml \)) along with various concentrations of MTX. Before these cells could synthesize thymidylate, purines, and glycine, FA from the medium had first to be transported into the cells and reduced to form an active FH\(_4\) pool. MTX has been shown to inhibit the reduction of FA (4), and we would therefore expect the growth of these cells to be inhibited in all 3 media.

The results of the experiment are shown in Chart 3. The concentrations required for inhibition in -T and -A media were about 10 times lower than those required to inhibit growth of cells containing an intracellular folate pool in the same medium (see Chart 2). Growth in -G medium, on the other hand, was inhibited by similar concentrations of MTX in cells with or without an intracellular folate pool; but cells with the pool (Chart 2) grew by 5- to 7-fold before growth ceased, whereas folate-depleted cells (Chart 3) stopped growing abruptly. The role of this folate pool in MTX toxicity will be discussed later.

Most of the experiments described above involved prolonged exposure of cells to MTX, and it is possible that some of the effects seen were the result of cellular metabolism of the drug. To examine this possibility, cells were grown for 48 hr in the presence of \(^3\)H-labeled MTX which was then extracted and analyzed chromatographically by procedures similar to those described for cellular folates. The \(^3\)H activity was quantitatively recovered, and no detectable change in the chromatographic properties of the labeled MTX was observed suggesting that significant metabolism of the drug had not occurred.

**DISCUSSION**

In contrast to the prediction made based on the single-site-of-action model of MTX action discussed in the introduction, the distribution of intracellular folate derivatives was the same in cells grown both in the presence and the absence of MTX and, in particular, no detectable
Action of MTX

accumulation of dihydro derivatives was observed. Our failure to see FH₄ accumulation is consistent with 3 possibilities: (a) that no FH₄ was produced by thymidylate synthetase because this enzyme was itself inhibited by MTX (3, 6); (b) that no FH₄ was produced because thymidine from the medium was phosphorylated to give rise to TMP concentrations high enough to cause inhibition of thymidylate synthetase; or (c) that FH₄ continued to be reduced in the presence of MTX. Our other experiments (see below) appear to favor Possibility a although Possibility c is consistent with observations of Condit and Mead (4), who observed that FH₄ could be reduced in vivo at MTX concentrations much higher than those required to inhibit the reduction of FA.

The growth experiments were undertaken to determine the mechanism(s) of MTX-induced inhibition of glycine, purine, and thymidylate synthesis. The kinetics of growth inhibition by MTX in -G medium were different from the inhibition in -T and -A media. This result suggested that the mechanism of MTX-induced inhibition of glycine synthesis was different from the mechanism(s) of purine and thymidylate inhibition. We shall first attempt to elucidate the mechanism of inhibition in -G medium.

The 5- to 7-fold increase in cell number seen in -G medium (Chart 2) was abolished if the folate pool in cells was previously depleted (see Chart 3). We have previously observed that if cells growing in medium containing FA were washed and resuspended in medium lacking both the vitamin and glycine, growth did not cease abruptly but continued until a 5- to 7-fold increase in cell number had occurred. Our explanation for this residual growth was as follows. For cells cultivated in medium lacking FA, the intracellular pool was the only source of folates, and this pool must have decreased as the cells grew and divided. When the intracellular folate concentration reached a level too low to support the synthesis of sufficient quantities of glycine, growth ceased due to partial glycine starvation. The fact that a 5- to 7-fold increase in cell number occurred in -G medium containing FA and MTX suggests that MTX inhibited entry of exogenous FA into the active cellular FH₄ pool. This effect of MTX has been shown before (4), but as far as the conversion of serine to glycine is concerned, MTX did not have any effect on the folate pool already in the cell.

As shown in Chart 2, high MTX concentrations brought about immediate inhibition of cell growth in -T and in -A media. Since the distribution of intracellular folate derivatives was not altered by the MTX concentrations used (Chart 1) and since FH₄ was available and functional for conversion of serine to glycine, it seems likely that MTX caused inhibition of thymidylate and purine synthesis by interfering directly with thymidylate synthetase and 1 or both folate-dependent enzymes involved in purine biosynthesis. Similar conclusions were reached by Borsa and Whitmore (2) who demonstrated, in fact, that thymidylate synthetase was inhibited in vitro by MTX (3).

The results of the experiment reported in Chart 3 indicate that MTX-induced inhibition of the conversion of FA to FH₄ occurred at MTX concentrations about 10 times lower than those required for the direct inhibition of thymidylate and purine synthesis. Although low concentrations of MTX inhibit the conversion of FA to FH₄, this effect is of little biological significance to cells exposed to MTX at high concentration for a short period of time because the cells contain an intracellular folate pool large enough to support growth for 48 hr. Thus for chemotherapeutic purposes, the MTX-induced inhibition of the conversion of FA to FH₄ produces no cell killing. It has been repeatedly observed (1, 2) that the important effect of MTX which produces cell death involves the inhibition of thymidylate synthesis. Both in vivo and in vitro (3, 6) evidence suggests that MTX inhibits thymidylate synthetase directly and that this effect, along with the direct inhibition by MTX of one of the purine synthesis enzymes, is the primary site of MTX action in vivo.

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