Consequences of Methotrexate Inhibition of Purine Biosynthesis in L5178Y Cells

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SUMMARY

Addition of 1 μM methotrexate to cultures of L5178Y cells results in an initial inhibition of thymidine, uridine, and leucine incorporation into acid-insoluble material followed, after about 10 hr, by a partial recovery in the extent of incorporation of these precursors. Acid-soluble adenine triphosphate and guanosine triphosphate concentrations are greatly reduced initially, but guanosine triphosphate concentrations appear to recover partially by 10 hr. Acid-soluble uridine triphosphate and cytidine triphosphate concentrations initially increase after methotrexate treatment but then, with time, they too decline. Hypoxanthine and guanine are more effective than is adenine in overcoming the methotrexate-induced inhibition of thymidine incorporation. These results suggest that, in the presence of methotrexate, guanine nucleotides become limiting for nucleic acid synthesis before adenine nucleotides do. The block of purine de novo synthesis in L5178Y cells by methotrexate is almost complete and is not reversed with time. This suggests that the additional purine nucleotides that are available for nucleic acid synthesis 8 to 10 hr after addition of methotrexate are being derived from nucleic acid breakdown. Consistent with this is the observed reduction in the number of polyribosomes and hence, presumably, in messenger RNA levels.

INTRODUCTION

Early work on the mechanism of action of methotrexate clearly showed that this folate antagonist interfered with the synthesis of purines, glycine, and thymidylate (5, 11, 12, 14, 18). The growth-inhibitory effects of methotrexate in mamalian cell cultures were overcome or prevented by the addition of thymidine, a purine, and, in some cases, glycine. That glycine was required only when dialyzed serum was used in culture media suggests that sufficient glycine ordinarily is supplied in undialyzed serum (5).

More recently, it has become generally acknowledged that inhibition of thymidylate biosynthesis by methotrexate is the key event leading to cell death (8). However, it has recently been shown that the cytotoxicity of methotrexate against cultured mouse lymphoma L5178Y cells is in part attributable to a "purineless" state (6, 7). Thus, hypoxanthine partially prevented the methotrexate-induced inhibition of thymidine, uridine, and leucine incorporation into macromolecules and also delayed the loss of cell viability, as measured by cloning experiments. These studies also showed that, during incubation of L5178Y cells with methotrexate in the absence of hypoxanthine, incorporation of thymidine into DNA was first inhibited but later partially recovered. It was suggested that inhibition of thymidine incorporation was due to lowering of purine deoxyribonucleotide concentrations, because of the methotrexate inhibition of purine de novo synthesis, and that the observed recovery of thymidine incorporation might be the result of an increase in synthesis of purines, due to reversal of the initial inhibition of purine synthesis or to degradation of nucleic acids within the cell with partial reuse of nucleic acid purines.

There have been several recent studies of the effect of methotrexate on deoxyribonucleotide concentrations (3, 16). However, although the methotrexate-induced inhibition of the incorporation of radioactive glycine and formate into cellular purines has been well documented (12, 13, 18), there appears to have been no study of the effect of this drug on intracellular concentrations of purine and pyrimidine ribonucleotides. The purpose of the present study was to study in greater detail the consequences of inhibition of purine biosynthesis de novo by methotrexate. Of particular interest were effects of methotrexate on purine and pyrimidine ribonucleotide concentrations and the possible relationship of such changes to previously observed effects of methotrexate on the synthesis of DNA, RNA, and protein.

MATERIALS AND METHODS

Fischer's medium and horse serum were obtained from Grand Island Biological Co., Grand Island, N. Y. [methyl-3H]Thymidine (2 Ci/m mole), [5-3H]uridine (25 Ci/m mole), and [U-14C]glycine (92 mCi/m mole) were obtained from New England Nuclear, Boston, Mass., while [4,5-3H]leucine (6 Ci/m mole) was from Schwarz/Mann, Orangeburg, N. Y. Nucleosides and bases were obtained from the Sigma Chemical Co., St. Louis, Mo. Methotrexate (sodium salt) was from Lederle Laboratories, Pearl River, N. Y. All
other reagents were of reagent grade and were obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J.

Mouse lymphoma L5178Y cells were grown in suspension culture in Fischer's medium and 10% horse serum (2). Stock cultures were diluted daily to about 20,000 cells/ml, and all the experiments described were carried out at cell concentrations between 80,000 and 125,000 cells/ml. Under these conditions the cell doubling time was between 10 and 12 hr.

To measure thymidine incorporation, 1- or 2-ml aliquots of cell culture were placed in stoppered 13- x 100-mm test tubes in a 37° water bath. [3H]Thymidine was added to each tube (1 μCi/ml), and at 5, 10, 15, and 20 min the contents of each tube were added to 5 volumes of 5% perchloric acid. The acid-insoluble fraction was washed 3 times with 5% perchloric acid and dissolved in 0.5 ml NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.), and the amount of the radioactivity incorporated was determined by liquid scintillation counting. The incorporation of [3H]thymidine into acid-insoluble material was linear for at least 20 min. Incorporation of [3H]leucine and [3H]uridine was determined in a similar manner, except that 5 and 2 μCi of isotope per ml, respectively, were used.

Concentrations of acid-soluble nucleotides were measured by high-pressure liquid chromatography of neutralized perchloric acid cell extracts, essentially as described previously (15).

Purine synthesis de novo was measured by determining the extent of [14C]glycine incorporation into total cellular adenine and guanine. Preliminary experiments had shown [14C]glycine incorporation to be linear for at least 90 min. Therefore, 30 min after adding [14C]glycine (1 μCi/ml, 12 μM) to the cell culture (20 to 80 ml), the cells were collected by centrifugation, and 1 ml of 0.4 M perchloric acid was added to the cell pellet. The stoppered tubes were heated at 100° for 1 hr. After centrifugation, the supernatant was applied to a 5- x 20-mm column of Dowex 50 (H+). The column was washed with three 10-ml portions of 0.1 N HCl and finally with 10 ml of 6 N HCl. Glycine is eluted with the 1st 0.1 N HCl wash, hypoxanthine is eluted with the 3rd 0.1 N HCl wash, and adenine, guanine, and 4-aminopurine are eluted with the 6 N HCl wash. The various fractions were evaporated to dryness and redissovled in 0.5 ml of water, and 0.1 ml was chromatographed (descending) on Whatman no. 3MM paper using 0.02 M imidazolecarboxamide are eluted with the 6 N HCl wash, and adenine, guanine, and 4-aminopurine are eluted with the 6 N HCl wash. The various fractions were evaporated to dryness and redissovled in 0.5 ml of water, and 0.1 ml was chromatographed (descending) on Whatman no. 3MM paper using 0.02 M imidazolecarboxamide are eluted with the 6 N HCl wash, and adenine, guanine, and 4-aminopurine are eluted with the 6 N HCl wash. The various fractions were evaporated to dryness and redissovled in 0.5 ml of water, and 0.1 ml was chromato-graphed (descending) on Whatman no. 3MM paper using either isopropyl alcohol:2 N HCl (65:35) or methanol:formic acid:water (70:15:5).

Analyses of ribosomes and polysomes by sucrose density centrifugation and of DNA by alkaline sucrose density centrifugation were carried out by published procedures (9, 10).

RESULTS

Prior to studying the effects of methotrexate on purine metabolism, it was necessary to establish that the methotrexate-induced inhibition of thymidine, uridine, and leucine incorporation into macromolecules was not due to lowered glycine concentration following inhibition of glycine synthesis by methotrexate. This was an especially important question because the Fischer's medium used contains no glycine. Table 1 shows that the addition of glycine to the medium to a final concentration of 10−4 M had no significant effect on the methotrexate-induced inhibitions. Presumably, the glycine requirement that the L5178Y cells should have in the presence of methotrexate is being met by glycine present in the horse serum. The L5178Y cells used in these experiments do not grow well in media containing dialyzed horse serum, and thus this point could not be studied further. Table 1 also shows that the extent of inhibition by methotrexate of thymidine and uridine incorporation into macromolecules is less at 8 and 10 hr than at 4 hr. These results confirm earlier observations (6) concerning partial recovery from some of the effects of methotrexate and show that the cells used in this study respond in the same way as those used previously.

In light of the observation that uridine incorporation into DNA recovered at the same time as did the thymidine incorporation, it was initially assumed that the thymidine being incorporated during the recovery period was representative of normal DNA synthesis. To test this assumption, we examined the distribution of the incorporated [3H]thymidine by alkaline sucrose density centrifugation of DNA. These studies showed there to be no difference between the control and the 12-hr methotrexate-treated cells. Such evidence is consistent with the initial assumption but does not rule out the possibility that the relative rates of DNA replication and DNA repair change during methotrexate exposure.

Since the recovery in thymidine incorporation into DNA is accompanied by a partial recovery in uridine incorporation into RNA, it had been suggested that both recoveries are due to increased availability of purines for polynucleotide synthesis (6, 7). To test this possibility, the effect of methotrexate treatment on the concentrations of several purine and pyrimidine ribonucleotides was measured directly. Results are shown in Chart 1. As expected, concentrations of adenine and guanine nucleotides decrease in the presence of methotrexate. There is, however, an increase in

<table>
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<th>Incorporation (% of control) at</th>
<th>2 hr</th>
<th>4 hr</th>
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<th>10 hr</th>
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<td>[3H]Thymidine</td>
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<td>11</td>
<td>23</td>
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<td>20</td>
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<tr>
<td>[3H]Uridine</td>
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<td>3</td>
<td>3</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>+ methotrexate and glycine</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>16</td>
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<tr>
<td>[3H]Leucine</td>
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<td>+ methotrexate</td>
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</tr>
<tr>
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<td>86</td>
<td>57</td>
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</table>
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GTP levels between 8 and 10 hr after addition of methotrexate, which corresponds with the time of the observed partial recovery of thymidine and uridine incorporation. The initial increase in UTP concentrations (which was also paralleled by increased UDP-glucose concentrations) was somewhat unexpected. Similarly, CTP concentrations also increased initially, but to a lesser extent. The CTP peak on the chromatogram was difficult to quantitate, as it was not completely resolved from the much larger UTP peak. The data of Chart 1 suggest that the partial recovery of thymidine and uridine incorporation observed after 8 to 10 hr of methotrexate exposure might be the result of increased guanine nucleotide concentrations.

Hypoxanthine has previously been shown to partially prevent the methotrexate-induced inhibition of thymidine, uridine, and leucine incorporation into macromolecules (6), and therefore the effects of hypoxanthine on purine and pyrimidine ribonucleotide concentrations were studied. Chart 1 shows that the intracellular concentrations of ATP and GTP are somewhat elevated when L5178Y cells were grown in the presence of $1 \times 10^{-4}$ M hypoxanthine. The presence of hypoxanthine in the medium eliminated the methotrexate-induced reduction in ATP and GTP concentrations and the transient increases in pyrimidine ribonucleotide concentrations. Furthermore, ATP and GTP levels were higher in the presence of methotrexate and hypoxanthine than in the presence of hypoxanthine alone.

The data of Chart 1 suggested that guanine nucleotides might become limiting for polynucleotide synthesis before adenine nucleotides did. If such were the case, guanine should be as effective as hypoxanthine in reversing the methotrexate-induced inhibition of DNA and RNA synthesis. On the other hand, adenine would be expected to be somewhat less effective, as we have previously shown that there is relatively little conversion of adenine to guanine ribonucleotides when L5178Y cells are incubated for 30 min with $[1^4C]$adenine (15). Chart 2A shows that, when the purine bases are added at the same time as $[1^4C]$thymidine, hypoxanthine and guanine partially reverse the inhibition of thymidine incorporation caused by a 4-hr exposure to methotrexate. Adenine is seen to be less effective. The purine bases were added at the same concentration, but the horse serum possesses a guanine deaminase and the half-life of the added guanine is only about 30 min. Xanthine had no effect on the inhibition of thymidine incorporation. Chart 2B shows that adding the purines 30 min prior to the addition of the thymidine results in more extensive reversal with hypoxanthine and guanine; adenine now also stimulates thymidine incorporation. The purine-deficient state caused by methotrexate is seen to be corrected, more efficiently, as far as thymidine incorporation is concerned, by hypoxanthine and guanine than by adenine. This is consistent with the idea that, during the decrease in purine nucleotide concentrations caused by methotrexate, guanine nucleotides become limiting for polynucleotide synthesis before adenine nucleotides do.

One possible basis for the partial recovery of guanine ribonucleotide pools during methotrexate treatment might be that the methotrexate-induced inhibition of purine biosynthesis de novo was being reversed with time. This hypothesis was tested directly by measuring the incorporation of $[1^4C]$glycine into total cellular purines as a function of time of exposure to methotrexate. The data of Chart 3 show that glycine incorporation into adenine and guanine is reduced to less than 5% after 2 hr of exposure to methotrexate and that the inhibition is not reversed. In subsequent experiments, using 5 times as many cells, it was possible to show that the extent of glycine incorporation into purines was still decreasing at the time of the observed increase in thymidine and uridine incorporation. For instance, at 4 and 10 hr after methotrexate addition, the $[1^4C]$glycine incorporation into cellular guanine was 2.5 and 1.6% of control, and incorporation into cellular adenine was 4 and 2%, respec-
tively. These experiments also showed that, in the presence of methotrexate, there was no accumulation of radioactive 4-amino-5-imidazole carboxamide or hypoxanthine derivatives. Thus it can be concluded that the partial recovery in thymidine incorporation observed 10 hr after addition of methotrexate is not due to a lessening of the inhibition of purine de novo synthesis.

The inhibition of protein synthesis produced by methotrexate in L5178Y cells is not as pronounced as its inhibition of DNA or RNA synthesis (Table 1). Since this inhibition is not the result of a glycine deficiency, it is probably the result of the lowered concentration of acid-soluble purine ribonucleotides. Lowered levels of ATP and GTP may affect protein synthesis directly, since these triphosphates are required for amino acid activation and for chain initiation and elongation, or indirectly, in that mRNA synthesis is curtailed and the existing mRNA has a limited lifetime.

Chart 4 shows that, in the presence of methotrexate, there is a significant decrease in the number of polyribosomes together with an increase in the number of single 80 S ribosomes. The addition of 0.1 mM hypoxanthine 30 min prior to the addition of methotrexate completely prevents this shift from polyribosomes to single 80 S ribosomes. This observed shift may reflect either decay of already synthesized mRNA, or inhibition of the initiation process, or both.

DISCUSSION

The present studies of the effects of methotrexate on acid-soluble purine ribonucleotide concentrations of L5178Y cells growing in culture do much to explain previous observations on the effects of methotrexate on macromolecular synthesis in this system (6, 7). The severe depletion of ATP and GTP gives rise to a purine-deficient state that results in an inhibition of RNA synthesis and, in conjunction with the block in thymidylate synthesis, curtails DNA synthesis. The inhibition of protein synthesis in this system is not the result of glycine deficiency but appears to be related to the limited lifetime of mRNA under conditions in which no replacement synthesis is occurring. The data presented also suggest that guanine nucleotides become limiting for nucleic acid synthesis before adenine nucleotides; this might have been expected, based on the relative concentrations of acid-soluble ATP and GTP. The partial recovery in thymidine incorporation that occurs some 8 to 10 hr after the initiation of methotrexate treatment appears to be related to an increased availability of acid-soluble purine nucleotides, particularly guanine nucleotides, which presumably arise from nucleic acid breakdown since purine biosynthesis de novo is still almost completely inhibited. The apparent reduction in mRNA content, as evidenced by the amount of polyribosomes present, indicates that the degradation of this nucleic acid species may be contributing acid-soluble precursors for reutilization. Further studies on nucleic acid breakdown in the presence of methotrexate are in progress.

The observed increases in concentrations of pyrimidine ribonucleotides when purine biosynthesis de novo is inhib-
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Very little is known about the undoubtedly complicated chain of events that lead to cell death after exposure to a cytotoxic agent. It may well be that the methotrexate-induced chain of events leading to cell death differs, depending on whether exogenous thymidine or purines are available to the cell population in question. Within this context it would be interesting to have more information regarding serum purine and pyrimidine concentrations in patients who respond and those who do not respond to methotrexate.

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REFERENCES


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