(6R)-5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid Effects on Nucleotide Metabolism in CCRF-CEM Human T-Lymphoblast Leukemia Cells

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ABSTRACT

(6R)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid [6R]DDATHF is a folate antimetabolite with activity specifically directed against de novo purine synthesis, primarily through inhibition of glycaminide ribonucleotide transformylase. This inhibition resulted in major changes in the size of the nucleotide pools in CCRF-CEM cells. After a 4-h incubation with 1 µM (6R)DDATHF, dramatic reductions in the ATP and GTP pools were observed, with almost no effect on CTP, UTP, and deoxyribonucleotide pools. When the incubation was continued in drug-free medium, recovery of ATP and GTP pools was protracted. ATP did not return to normal until 24–36 h, and GTP pools were only partially repleted by 48 h. The ATP and GTP pools were not affected when the initial 4-h incubation with (6R)DDATHF was conducted in the presence of 100 µM hypoxanthine. Addition of hypoxanthine to the medium after a 4-h incubation with (6R)DDATHF caused rapid recovery of the ATP and GTP pools. Similar effects were seen when the purine precursor aminoimidazole carboxamide was used in place of hypoxanthine. The effect of (6R)DDATHF on nucleotide pools and the capability of hypoxanthine or aminoimidazole carboxamide to prevent or reverse this phenomenon correlated directly with the inhibition of cell growth. Presumably as a consequence of the decrease in purine nucleotide triphosphate levels, the conversion of exogenously added uridine, thymidine, and deoxyuridine to nucleotides was markedly decreased. These effects were protracted for almost 48 h and were also reversed by hypoxanthine. Differential repletion of ATP and GTP pools after (6R)DDATHF pretreatment demonstrated that diminished precursor phosphorylation is primarily a consequence of GTP rather than ATP starvation.

INTRODUCTION

5,10-Dideaza-5,6,7,8-tetrahydrofolic acid is a representative of a new class of antimetabolites designed as inhibitors of folate-dependent enzymes other than dihydrofolate reductase (1). DDATHF is a potent inhibitor of cell growth in culture (2) and also displays broad spectrum activity against transplantable murine solid tumors and human tumor xenografts (3). The 6R diastereomer of DDATHF (Lometrexol) is currently undergoing phase I clinical trials. Previous biochemical studies have determined the primary site of DDATHF action to be inhibition of glycaminide ribonucleotide transformylase, an early step in de novo purine biosynthesis. These studies also demonstrated that inhibition of neither dihydrofolate reductase nor TS was involved in the action of DDATHF (2). Depletion of intracellular purine nucleotide pools was also shown to result from the action of DDATHF. In this report we explore more fully the relationship between the effects of (6R)DDATHF on de novo purine biosynthesis, the consequent changes in intracellular nucleotide pools, and the effects on cell metabolism and proliferation.

MATERIALS AND METHODS

DDATHF diastereomers, differing in configuration about C-6, were kindly supplied by Dr. Chuan Shih, Lilly Research Laboratories (4, 5). A solution of the sodium salt was used and concentrations were determined by using an extinction coefficient of 9.15 × 10² M⁻¹ cm⁻¹. [5⁻³H]-2-Deoxyuridine (23 Ci/mmol) and [2,8⁻³H]dATP (24 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [methyl-3H] Thymidine (40 Ci/mmol), [6⁻³H]deoxyuridine (16.6 Ci/mmol), and [2,4⁻¹⁴C]uridine (50 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). [methyl-³H]dTTP (21 Ci/mmol) was obtained from NEN Research Products (Boston, MA). Mycophenolic acid was a gift from Dr. A. C. Sartorelli (Yale University, New Haven, CT). All other chemicals were from Sigma (St. Louis, MO) or Schwarz-Mann (Cleveland, OH) and were the highest purity available.

Media, sera, and antibiotics for tissue culture were purchased from Gibco (Grand Island, NY). Plasticware was purchased from Corning Glass Works (Corning, NY).

Cell Culture. A cloned subline of the human T-lymphoblast cell line CCRF-CEM was used (6). Cells were grown and treated in suspension culture in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a 5% CO₂ atmosphere. Every 3 months stocks were checked for Mycoplasma contamination by using the gene probe method (Gene-Probe, San Diego, CA) and were found Mycoplasma free.

Ribonucleotide Pool Determination. Cells (5–10 × ¹⁰⁶) were collected and extracted with 1 M formic acid saturated with n-butyl alcohol on ice (7). The extract was lyophilized and reconstituted with mobile phase before high performance liquid chromatography analysis. Separation of nucleotides was performed on a Whatman Partisil-10 SAX anion exchange column (Whatman, Woburn, MA), using 0.4 M ammonium phosphate isocratic elution at a flow rate of 1.5 ml/min (8). Eluted nucleotide triphosphates were monitored with a Model 153 Altex (Berkeley, CA) detector set at 254 nm and the retention peak areas were determined with a Shimadzu C-RIA Chromatopac Integrator (Kyoto, Japan).

Deoxyribonucleotide Pool Determination. Cells (2 × ¹⁰⁶) were washed twice with ice-cold phosphate buffered saline and extracted with 0.5 N PCA. The supernatant was then neutralized with 1 M KH₂PO₄, pH 7.5, and 5 N KOH and stored at −70°C until analysis. Pool sizes were determined by using a DNA polymerase assay as described by Williams et al. (9).

[³H]Nucleoside Uptake. For the determination of transport and uptake into acid soluble fraction, aliquots of cell suspensions were placed in rapid sampling tubes consisting of 400-µl microfuge tubes containing 120 µl of oil (a 16:84 mixture of light paraffin oil and Dow Corning 550 silicone fluid) layered over 50 µl of 15% TCA. The cells were pelleted into the TCA layer by centrifugation for 2 min at 10,000 × g (10). The cell pellet was washed twice with 15% TCA and the supernatants were counted for radioactivity together with the supernatant obtained from the initial centrifugation.

[³H]Nucleoside Incorporation. Cells, at a density of 4–6 × ¹⁰⁵ cells/ml, were incubated with 1 µCi (6R)DDATHF for 4 h. After treatment, the cells were harvested by centrifugation (1000 × g for 5 min at 37°C), washed twice with complete medium, and resuspended in drug-free medium, with or without other agents (hypoxanthine, adenine, guanine, mycophenolic acid) at approximately the initial density. At appropriate times, 50 ml of the cell suspension were centrifuged and the cells were
resuspended in 2 ml of medium, after which [methyl-3H]thymidine, [6-3H]deoxyuridine, or [2-'4C]uridine was added.

At intervals duplicate aliquot portions were removed, washed, and the macromolecular species were precipitated by adding 2% PCA. The precipitate was washed twice with 2% PCA and resuspended in 10% PCA. The suspension was boiled for 15 min, centrifuged, and the radioactivity was determined (11). Results were expressed as cpm/10^6 cells. Both the inhibition by (6R)DDATHF and the rescue by hypoxanthine, adenine, and guanine caused changes in the specific activity of the nucleotide pools compared. Specific activities of these pools were calculated by using the measured values for radioactivity in the acid soluble fraction and the total size of the nucleotide pool. These adjusted specific activities were then used to calculate corrected values for the synthetic rates of the macromolecular species. The extent of inhibition of nucleoside incorporation produced by (6R)DDATHF was calculated by comparing the slopes obtained from the least squares linear regression analysis of the inhibited and control incorporation data.

[5-3H]-2'-Deoxyuridine Tritium Release Assay. Inhibition of TS in intact cells was measured by estimation of tritium released after cellular uptake of [5-3H]-2'-deoxyuridine conversion to [5-3H]dUMP, and the subsequent release of 3H2O during the TS reaction which replaced the 5-3H with a methyl group (12). Cells were collected by centrifugation and suspended in 1 ml of medium at a concentration of 0.5-1.0 x 10^6 cells/ml. After addition of [5-3H]deoxyuridine (0.1 μCi/ml), aliquots of 100 μl were taken at intervals and the reaction was stopped by addition of 200 μl of a suspension of 10% activated charcoal in 4% trichloroacetic acid. After centrifugation, 200 μl of supernatant were measured for radioactivity. Results were analyzed as described above.

RESULTS

Our previous studies (2) measured ATP and GTP levels only at a single time point after prolonged exposure (6 h) of cells to DDATHF. We therefore conducted a more thorough analysis of the time course of the effects of (6R)DDATHF on intracellular nucleotide pools. As shown in Fig. 1, when CCRF-CEM cells were incubated with 1 μM (6R)DDATHF, an effect on nucleotide pools became evident after 2 h in the presence of the drug. At the end of 4 h of incubation, ATP and GTP pools were decreased by 40–50%. There was a slight drop of CTP levels, limited to 10–15%, and some increase in UTP pools. Cells were then transferred to drug-free medium and, after an additional 4-h incubation in drug-free medium, both ATP and GTP dropped further to 30–35% of control. The ATP level remained stable for at least 24–36 h, while there was a slow recovery of the GTP pool to 50–60% of the size of an untreated control after 24–48 h.

This dramatic effect on adenine and guanine nucleotide pools was completely reversed by the addition of 100 μM hypoxanthine. This was true whether hypoxanthine was added simultaneously with (6R)DDATHF or when, after 4 h of exposure to (6R)DDATHF, cells were resuspended in drug-free medium with added hypoxanthine (Fig. 2, A and B). In addition to the augmentation of ATP and GTP pools by hypoxanthine, decreases in the UTP and CTP pools were also observed. This decrease, as shown in Fig. 2C, was due only to the effect of hypoxanthine. The reversal of (6R)DDATHF effects by hypoxanthine was seen not only in the size of ATP and GTP pools (Fig. 3) but also in cell growth inhibition (Fig. 4). The purine precursor aminomimidazole carboxamide also protected against (6R)DDATHF suppression of nucleotide pools and cell growth (Figs. 3 and 4). For both aminomimidazole carboxamide and hypoxanthine, the concentration dependence for reversal of the nucleotide pool effects was concordant with that for protection from the growth inhibitory effects of (6R)DDATHF.

Deoxyribonucleotide pools were determined after 4 h of incubation with 1 μM (6R)DDATHF and additional 4 h in drug-free medium, a time when ATP and GTP reach a minimal level. dATP and dGTP pools were not decreased at this time, rather, their size was increased approximately 50% compared to an untreated control. The addition of hypoxanthine following (6R)DDATHF doubled the size of dATP and dGTP pools (Table 1).

(6R)DDATHF at concentrations up to 100 μM, did not have inhibitory effects on TS in vitro using either Lactobacillus casei purified enzyme, as previously reported, or a crude extract from CCRF-CEM cells as assayed according to the isotopic method of Roberts (13). This finding was consistent with the observation that thymidine did not prevent DDATHF cytotoxicity in the L1210 and CCRF-CEM cell lines (2). However, CCRF-CEM cells assayed for in situ TS activity after a 4-h treatment with 1 μM (6R)DDATHF showed markedly diminished 3H2O release from [5-3H]deoxyuridine (Fig. 5). This effect was maximum 4 h after completing (6R)DDATHF exposure, falling to
ATP pool; B, GTP pool; (•) hypoxanthine; (O) aminoimidazole carboxamide.

1. (6R)DDATHF..1. then washed and resuspended in drug-free medium in the presence of different concentrations of hypoxanthine or aminoimidazole carboxamide. Levels falling to 25 and 34%, respectively, of untreated control. (•) hypoxanthine; (O) aminoimidazole carboxamide; (A) (6R)DDATHF alone.

about 25% of the untreated value, and was followed by a slow recovery after 8–24 h in drug-free medium. The same treatment caused even more dramatic suppression of [methyl-3H]thymidine and [6-3H]deoxyuridine incorporation into macromolecules (Fig. 5). By 4 h following drug exposure, thymidine and deoxyuridine incorporation were reduced to 8 and 4% of untreated control values, respectively. These results differed from our short term (30 min) exposure studies, in which no suppression of thymidine or deoxyuridine incorporation was found as an immediate consequence of incubation with (6R)DDATHF. At this time intracellular purine nucleotide pools are minimally perturbed (Fig. 1). In the present study cells were incubated with (6R)DDATHF for 4 h before being analyzed for nucleotide incorporation. The marked diminution in intracellular ATP and GTP pools which takes place after this degree of drug exposure could alter the apparent rates of a number of processes involving nucleotide metabolism.

A marked decrease in the uptake of [3H]thymidine and [14C]deoxyuridine into the intracellular acid soluble species (nucleotides) was seen after 4 h of exposure to (6R)DDATHF, with levels falling to 25 and 34%, respectively, of untreated control cells. The early time course of uptake (<2 min) in CCRF-CEM cells of either [3H]thymidine or [14C]uridine in the presence of 1 μM DDATHF did not show any alteration in the transport of these precursors of nucleic acids (data not shown). The diminished formation of nucleotides at later time points, however, strongly suggested decreased nucleoside phosphorylation as the actual cause of the apparent suppression of 3H2O release and decreased macromolecular incorporation. This prompted further investigation into the comparative effects of the sizes of the ATP and GTP pools on the overall process of nucleoside phosphorylation.

The incubation of CCRF-CEM cells after 1 μM (6R)DDATHF with 100 μM adenine restored both ATP and GTP pools to normal values with concomitant recovery of the apparent rate of [3H]thymidine incorporation into DNA and [14C]Urd incorporation into RNA. DNA and RNA incorporation were also returned to normal when the rescue agent was 100 μM guanine. Under these conditions, only GTP returned to control levels and ATP did not show any change compared to cells treated with (6R)DDATHF and then left in drug-free medium (Table 1).

In order to replete only ATP pools, the cells were incubated, after (6R)DDATHF treatment, with medium containing 100 μM hypoxanthine and 50 μM mycophenolic acid, an inhibitor of inosinate dehydrogenase. In this case, ATP reached nearly normal values, GTP did not increase, and nucleic acid incorporation remained completely inhibited (Table 1).

DISCUSSION

The data presented demonstrate that a major consequence of exposure of CCRF-CEM cells to (6R)DDATHF is a marked diminution in the size of purine nucleotide pools. This depletion is time dependent and directly correlates with (6R)DDATHF cytotoxicity. As shown in Fig. 1, both ATP and GTP pools become greatly depleted after 4 h of drug exposure. Purine nucleotide pools depletion deepens further and is protracted for more than 48 h after removal of the drug. This suggests either a high affinity of (6R)DDATHF for the enzyme target and/or prolonged retention of the drug into the cells as might result from formation of (6R)DDATHF polyglutamates (14).

The results also demonstrate the capacity of hypoxanthine to block the effects of (6R)DDATHF on adenine and guanine nucleotide pools with a dose response correlating directly with its ability to prevent the growth inhibitory effects of (6R)DDATHF. This strongly implicates the decrease in purine nucleotide pools in the mechanism of (6R)DDATHF action. One consequence of this is demonstrated in the effects of (6R)DDATHF on the incorporation of DNA and RNA precursor nucleosides into acid soluble pools. This almost certainly results from a limitation in the supply of ATP and/or GTP to serve as phosphate donors in the kinase reactions necessary for conversion of the nucleosides to nucleotides. An effect due to a feedback inhibition of the kinase reactions by increased pyrimidine nucleotide pools is also possible, but seems unlikely, as the magnitude of the increase amounts to only 20–30%. It is highly likely that other ATP or GTP dependent reactions would be suppressed as well.

The diminished observed incorporation of radiolabeled precursors into DNA and RNA seems to reflect not only a lessened phosphorylation of labeled nucleosides to activated precursors, but also a diminished rate of macromolecular synthesis overall. A strong indication of the above mentioned effect is reported in Table 1, where there is a marked decrease in the macromolecular synthesis associated with a decrease in ATP and GTP pools even after correction for the specific activity changes in the nucleotide pools. Surprisingly, no decrease in the purine
Table 1  Ribo- and deoxyribonucleotide pools, and DNA and RNA synthesis in CCRF-CEM cells

The results are the average of three experiments conducted in duplicate, with SD less than 15%. Cells were incubated for 4 h with 1 μM (6R)DDATHF, then washed and resuspended in drug-free medium containing the above mentioned compounds. Ribo- and deoxyribonucleotide pools, and DNA and RNA synthesis were examined 4 h later as described in “Materials and Methods.” In control cells, the nucleotide concentrations were: CTP, 0.48 nmol/10⁶ cells; UTP, 1.63 nmol/10⁶ cells; ATP, 5.34 nmol/10⁶ cells; and GTP, 3.12 nmol/10⁶ cells; in the case of deoxyribonucleotides dATP, 60 pmol/10⁶ cells; dGTP, 36 pmol/10⁶ cells.

<table>
<thead>
<tr>
<th>% of control</th>
<th>[%H]TdR incorporated into DNA</th>
<th>[%H]Urd incorporated into RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP</td>
<td>UTP</td>
<td>ATP</td>
</tr>
<tr>
<td>Hypoxanthine, 100 μM</td>
<td>69.8</td>
<td>54.2</td>
</tr>
<tr>
<td>(6R)DDATHF, 1 μM</td>
<td>80.1</td>
<td>128.8</td>
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<tr>
<td>(6R)DDATHF + hypoxanthine</td>
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<td>68.7</td>
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<tr>
<td>(6R)DDATHF + adenine, 100 μM</td>
<td>75.0</td>
<td>60.6</td>
</tr>
<tr>
<td>(6R)DDATHF + guanine, 100 μM</td>
<td>95.8</td>
<td>103.1</td>
</tr>
<tr>
<td>(6R)DDATHF + hypoxanthine + MA</td>
<td>73.6</td>
<td>104.4</td>
</tr>
<tr>
<td>(6R)DDATHF + MA, 50 μM</td>
<td>85.6</td>
<td>204.5</td>
</tr>
</tbody>
</table>

* dThd, thymidine; ND, not determined.
* Corrected for specific activity as described in “Materials and Methods.”

Deoxyribonucleotide pools was found even when ATP and GTP pools were down to 30-35% of the control levels.

Differential repletion of ATP and GTP pools was used to determine which pool was primarily responsible for the effects on DNA and RNA incorporation. The addition of adenine to the media after exposure to (6R)DDATHF repleted both ATP and GTP pools and consequently DNA and RNA incorporation. When guanine was used, there was a complete recovery of the GTP pool, but the ATP pool remained depressed. Both DNA and RNA incorporation were completely resumed. Repeating only the ATP pool, using hypoxanthine and mycophenolic acid (15) resulted in nearly complete recovery in the size of the ATP pool, the GTP pool remained depressed. There was still a complete inhibition of DNA and RNA incorporation. These results demonstrate that the depletion of guanine ribonucleotide pool is primarily responsible for the inhibition of precursor phosphorylation and presumably DNA and RNA synthesis.

Further indications of a connection between the apparent rate of DNA synthesis and size of the GTP pool are apparent in the timing of recovery of thymidine and deoxyuridine incorporation into DNA after cessation of (6R)DDATHF exposure (Fig. 5). These are better correlated with the restoration of the GTP pool, which had returned to nearly 60% of the control value by 24 h, then with the ATP pool which returned toward normal more slowly (Fig. 1).

Recent reports have also noted a specific relationship between GTP levels and the ability of G proteins to function as transducers of intracellular signals, particularly their control on adenylate cyclase and cyclic guanosine 3',5'-monophosphate-specific phosphodiesterase (20-21). Disruption of these signal transduction pathways could certainly be part of the action of (6R)DDATHF.

In summary, the present results demonstrate the importance of depletion of purine nucleoside triphosphates as a consequence of inhibition of de novo purine biosynthesis in these cells. There is an absolute requirement for net purine synthesis in order to supply precursors for the new DNA and RNA synthesis necessary for cell growth and division. In the absence of a sufficient supply of purines via salvage pathways and a limited supply from de novo synthesis, we presume that purine triphosphate pools become depleted as a result of attempted DNA and RNA synthesis as well as ongoing irreversible oxidative purine catabolism. Intracellular purine economy eventually reaches a new equilibrium where there is little or no net DNA and RNA synthesis and the purine triphosphate pools are markedly diminished. A further consequence of this seems to be a lessened ability to carry out critical phosphorylation reactions as indicated by the decreased conversion of thymidine and deoxyuridine to their corresponding nucleotides. All the above provide a rational basis for understanding the mechanism of growth inhibition by inhibitors of de novo purine biosynthesis such as DDATHF.

REFERENCES


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