Contributions of the Depletions of Guanine and Adenine Nucleotides to the Toxicity of Purine Starvation in the Mouse T Lymphoma Cell Line

Marvin B. Cohen and Wolfgang Sadee

ABSTRACT

The importance of the depletion of guanine nucleotides in cellular toxicity was compared to that of the depletion of adenine nucleotides following the inhibition of early de novo purine biosynthesis in the mouse T lymphoma (S-49) cell line. Early de novo purine biosynthesis was blocked with 6-methylmercaptopurine ribonucleoside (6MMPR), while adenine and guanine nucleotide biosyntheses were individually inhibited using L-alanosine (alanosine) and mycophenolic acid (MA), respectively. Incubation with either alanosine or MA depleted adenosine 5′-triphosphate or guanosine 5′-triphosphate levels, respectively, to the same extent as those caused by 6MMPR. The effects of a 3-hr incubation with either 6MMPR or MA on nucleic acid synthesis were similar: a partial inhibition of RNA synthesis; and a dramatic inhibition of DNA synthesis. However, a 3-hr incubation with alanosine did not significantly affect either RNA or DNA synthesis. Furthermore, the effect of exogenous sources of adenine or guanine nucleotides on adenosine 5′-triphosphate and guanosine 5′-triphosphate levels, and DNA synthesis rates in cells pretreated with 6MMPR for 3 hr were studied. Resumption of DNA synthesis was dependent on the return of guanine nucleotide levels, but not of adenine nucleotide levels, to normal. Finally, MA and 6MMPR had the same effect on the progression of cells through the cell cycle, while the effect of alanosine was dramatically different. These results suggest that the biological consequences of purine starvation are primarily mediated by the depletion of guanine nucleotides rather than that of adenine nucleotides. We previously found that the depletion of guanine ribonucleotides rather than that of guanine deoxyribonucleotides is associated with the toxicity when guanine nucleotide biosynthesis is inhibited and that the depletion is associated with the inhibition of DNA synthesis. These combined results indicate that a function of guanine ribonucleotides, probably in DNA synthesis, is the cellular process most sensitive to the inhibition of de novo purine biosynthesis.

INTRODUCTION

The inhibition of de novo purine biosynthesis is one of the biochemical effects of two important drugs, methotrexate and 6-mercaptopurine, used clinically as antineoplastic and immuno-suppressive agents (10, 17, 19). The biochemical effects of these agents are complex and involve other aspects in addition to purine depletion. For example, the relative importance of purine starvation compared to that of dThd4 or glycine starvation caused by methotrexate is still not clear (13, 22, 27). Also, 6-mercaptopurine incorporates as 6-thioguanine into RNA and DNA (18, 23, 24) and, in prokaryotic cells, may disrupt DNA repair by inhibiting a 3′- to 5′-exonuclease (2, 3) in addition to depleting purine nucleotides. However, 6MMPR, a phosphorylated metabolite which inhibits pyrophosphate-ribose-phosphate amidotransferase, has no known modes of action other than the inhibition of de novo purine nucleotide biosynthesis, yet it is still toxic to certain transplantable tumors (1) and tumor cells in culture (1, 26, 28). Its toxicity is associated with the depletion of purine nucleotides resulting in growth inhibition and the inhibition of RNA and DNA synthesis (1, 26, 28).

The present study uses 6MMPR as a model compound to determine the relative importance of guanine and adenine nucleotide depletion to the toxicity resulting from purine starvation. For this purpose, we compare the biochemical effects of total purine starvation caused by 6MMPR to those caused by the specific depletion of guanine nucleotides by MA, an inhibitor of IMP dehydrogenase, and of adenine nucleotide depletion by alanosine, a metabolite of which is an inhibitor of adenylosuccinate synthetase (7). We present evidence that the biological consequences of purine starvation are dominated by those associated with guanine nucleotide depletion.

MATERIALS AND METHODS

Reagents and Apparatus. MA (NSC 129185) was provided by Eli Lilly and Co., Indianapolis, Ind. Alanosine was provided by Drug Research and Development, National Cancer Institute, Bethesda, Md. Nucleosides and nucleotides of analytical grade; alkaline phosphatase (orthophoric monoester phosphorylase; EC 3.1.3.2) type I, from Escherichia coli; deoxyribonuclease 5′-oligonucleotidohydrolase (EC 3.1.4.5) type I, from bovine pancreas; phosphodiesterase I (oligonucleate 5′-nucletidohydrolase, EC 3.1.4.1) type VI, from Crotalus adamanteus; and 6MMPR were purchased from Sigma Chemical Co., St. Louis. [U-14C]Cytidine (485 mCi/mmol) and NCS tissue solubilizer were purchased from Amersham, Inc., Arlington Heights, Ill. [methyl-3H]dThd (80 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

HPLC analyses were performed on a Model ALC/GPC 204 liquid chromatograph (Waters Associates, Milford, Mass.), cell density measurements were performed on a Model Z Coulter Counter (Coulter Electronics, Hialeah, Fla.), radioactive analyses were performed on a Beckman 9000LS liquid scintillation counter (Beckman Institute, Palo Alto, Calif.), and cellular DNA contents were measured on a Model FACS III fluorescence-activated cell sorter (Becton, Dickinson & Co., Mountain View, Calif.).

Cell Culture. Mouse T lymphoma (S-49) cells were grown in suspension at 37° and 10% CO2 in Dulbecco’s modified Eagle’s medium.
supplemented with 10% heat-inactivated horse serum. Their growth properties have been described previously (16).

Growth experiments were performed in Costar (24-well) tissue culture plates. Two ml of cells in complete medium (3 x 10^5 cells/ml) were added to the combination of agents to be tested. After 24 hr, cell density was measured. Percentage of growth is defined as:

\[(\text{Final treated density}) - (\text{initial density})\]  

\[(\text{Final control density}) - (\text{initial density})\]

Treatment was considered lethal when the final cell density was less than the initial density after a 24-hr incubation.

**Measurement of Intracellular Nucleotide Concentrations.** One hundred ml of cells at a density of approximately 10^6 cells/ml were incubated at 37° in the presence of the combination of agents to be tested for 3 hr. Two µCi of [U-14C]cytidine were added to the cells 30 min prior to harvest. The cells were pelleted by centrifugation, washed with phosphate-buffered saline, and extracted with 0.4 N perchloric acid. The extract was neutralized by ion pair extraction (14). The extract was stored frozen until analysis. The acid-insoluble pellet was washed with H2O and stored similarly. Concentrations of purine and pyrimidine ribonucleotides in the acid-insoluble extract were determined by HPLC using a Partisil-SAX anion-exchange column and a mobile phase of 0.4 M potassium phosphate, pH 3.6, at a flow rate of 2.0 ml/min.

**Analysis of RNA and DNA Synthesis Rates.** The rates of RNA and DNA synthesis were measured by the incorporation of [U-14C]cytidine into acid-insoluble material corrected for the specific activity of the appropriate precursor, i.e., CTP or dCTP (16). The acid-insoluble cell pellet was suspended overnight in 250 µl of 0.3 N KOH to hydrolyze RNA to free ribonucleotides. Acidification with 100 µl of 0.8 N HClO4 precipitated DNA while free ribonucleotides remained in the supernatant. The supernatant was adjusted to alkaline pH with 200 µl of 0.5 M Tris buffer, pH 8.5, and then incubated with 2 units of alkaline phosphatase for 2 hr at 37° to yield free ribonucleotides. The DNA pellet was suspended in 500 µl of 0.1 M Tris buffer, pH 8.5; 25 µl of 0.3 M MgCl2; and 200 units of DNase I, and incubated for 4 hr at 37°, after which 0.05 unit of phosphodiesterase I and 2 units of alkaline phosphatase were added, and the incubation was continued for 4 hr. Free nucleotides obtained from RNA and DNA were chromatographed by HPLC and analyzed for radioactivity in cytidine and deoxycytidine (16).

**Reversal of DNA Synthesis Inhibition by Purine Bases.** The ability of purine bases to reverse the DNA synthesis inhibition caused by 6MMPR was measured by the incorporation of [methyl-3H]dThd into DNA. Ten ml of cells (density, 8 x 10^6 cells/ml) were incubated with 3.0 µM 6MMPR for 3 hr. At time zero, 4 µCi of [3H]dThd (16 Ci/mmol) and 6MMPR was measured by the incorporation of [methyl-3H]dThd into acid-insoluble material corrected for the specific activity of the appropriate precursor, i.e., CTP or dCTP (16). The acid-insoluble cell pellet was suspended overnight in 250 µl of 0.3 N KOH to hydrolyze RNA to free ribonucleotides. Acidification with 100 µl of 0.8 N HClO4 precipitated DNA while free ribonucleotides remained in the supernatant. The supernatant was adjusted to alkaline pH with 200 µl of 0.5 M Tris buffer, pH 8.5, and then incubated with 2 units of alkaline phosphatase for 2 hr at 37° to yield free ribonucleotides. The DNA pellet was suspended in 500 µl of 0.1 M Tris buffer, pH 8.5; 25 µl of 0.3 M MgCl2; and 200 units of DNase I, and incubated for 4 hr at 37°, after which 0.05 unit of phosphodiesterase I and 2 units of alkaline phosphatase were added, and the incubation was continued for 4 hr. Free nucleotides obtained from RNA and DNA were chromatographed by HPLC and analyzed for radioactivity in cytidine and deoxycytidine (16).

**RESULTS**

**Toxicity of Guanine and Adenine Nucleotide Depletion.** The effects of 6MMPR, MA, and alanosine on the growth of S-49 cells are shown in Chart 1. The 50% growth inhibition values were approximately 0.8, 0.35, and 8.0 µM, respectively. Further experiments were conducted using a concentration of approximately 4 times the 50% growth inhibition of each compound: 3.0; 1.2; and 37.5 µM, respectively.

**Nucleotide Level Changes.** Incubation of S-49 cells with 3.0 µM 6MMPR for 3 hr reduced the concentrations of ATP and GTP to 58 and 30% of control, respectively, while the pyrimidine ribonucleotides, UTP and CTP, increased to 140 and 170% of control (Table 1). However, studies on the L5178Y cells found that 6 MMPR depleted ATP to the same extent that it depleted GTP (26, 28). MA (1.2 µM) decreased the GTP level to 22%, while the pyrimidine ribonucleotides increased, and ATP was relatively unaffected (Table 1). Incubation with 37.5 µM alanosine reduced the ATP level to 60% of control, while the GTP level almost doubled, and the pyrimidine ribonucleotides were unaffected (Table 1). A similar increase in GTP was found in an S-49 mutant cell line partially deficient in adenylsuccinate synthetase, the same enzyme inhibited by alanosine (25).

**DNA and RNA Synthesis Rates.** The relationship of DNA and RNA synthesis rates to the depletion of guanine and/or adenine nucleotides was studied. Between 2.5 and 3 hr after the addition of 3.0 µM 6MMPR, the rate of synthesis of DNA was 8% of that of control, and the rate of synthesis of RNA was 37% of that of control (Table 2). Similarly, upon incubation with 1.2 µM MA, the rates of DNA and RNA synthesis were reduced to 2 and 54% of that of control, respectively, during the same time period (Table 2). However, treatment with 37.5 µM alanosine had only a minimal effect on the rates of the synthesis of either RNA or DNA (Table 2).
2). Studies on Novikoff rat hepatoma (N1S1-67) cells showed minimal effects on nucleic acid synthesis after a 5-hr incubation with a toxic concentration of alanosine (7).

Reversal of DNA Synthesis Inhibition by the Addition of an Exogenous Supply of Adenine or Guanine Nucleotides. The incorporation of [3H]dThd into acid-insoluble material was linear over the 50-min period studied and, for this purpose, was considered an acceptable estimate of the relative rate of DNA synthesis. After a 3-hr pretreatment with 3.0 μM 6MMPR, [3H]dThd incorporation was reduced to <2%. Addition of 40 μM adenine, 200 μM adenine, or 40 μM guanine was not able to alleviate the DNA synthesis inhibition caused by 6MMPR (Chart 2). Only after addition of 200 μM guanine did DNA synthesis start to resume after approximately 20 min (Chart 2).

The nucleotide levels of cells incubated for 30 min with each of the above concentrations of guanine or adenine after a 3-hr pretreatment with 3.0 μM 6MMPR were measured (Chart 3). Both concentrations of adenine had similar effects on ATP levels, returning them to about 90% of control, despite the continued presence of 6MMPR. Neither concentration of adenine had any

table 2

<table>
<thead>
<tr>
<th>Nucleic acid synthesis (% of control)</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>6MMPR (3.0 μM)</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>Alanosine (37.5 μM)</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td>MA (1.2 μM)</td>
<td>54</td>
<td>2</td>
</tr>
</tbody>
</table>

effect on GTP levels. Likewise, neither concentration of guanine had any effect on ATP levels. However, 200 μM guanine returned GTP levels to normal, while 40 μM guanine had little effect (Chart 3).

Effects on Cell Cycle Progression. Cells treated with MA, alanosine, or 6MMPR were analyzed by flow cytometry to determine the effects of these agents on the progression of cells through the cell cycle. Cells incubated with either mycophenolic acid or 6MMPR accumulated with time at the G1-S interface of the cell cycle, while there was a drastic decrease in the percentage of cells in the late-S-G2-M portion of the cell cycle. Early S

![Chart 3. ATP and GTP levels during attempted reversal of purine starvation-induced DNA synthesis inhibition by purine bases. Cells were pretreated with 3.0 μM 6MMPR for 3 hr. At that time, either adenine or guanine was added (bars); ade, 40 μM; ADE, 200 μM. Nucleotide levels were measured 30 min later.](chart3.png)

![Chart 4. Changes in the cell cycle distribution of S-49 cells after exposure to 1.2 μM MA, 37.5 μM alanosine, or 3.0 μM 6MMPR. Abscissa, relative fluorescent intensity (DNA content); ordinate, relative number of cells. Percentage of cells in each phase of the cell cycle as determined by computer analysis is shown with Gs- M containing cells from very late S as described in "Materials and Methods."](chart4.png)
and late S were relatively unaffected. On the contrary, cells incubated with alanosine accumulated at a point well into early S, while the percentage of cells in G1 remained unchanged or decreased slightly. Unlike the previous compound, the percentage of cells in the later portion of the cell cycle did not decrease for at least 4 hr (Chart 4).

DISCUSSION

The independent inhibition of either de novo guanine nucleotide biosynthesis or de novo adenine nucleotide biosynthesis beyond the branching of the biosynthetic pathways, as well as the combined depletion of adenine and guanine nucleotides by inhibition of de novo purine biosynthesis at an early step, are toxic to cells (12–14, 20). Therefore, one might initially expect that the overall toxicity of purine starvation is a balanced composite of the toxicities resulting from the depletion of guanine and adenine nucleotides.

To allow the comparison of the biological consequences of MA and alanosine to those of 6MMPR, it was desired that the primary effects, i.e., nucleotide depletion, be equivalent. Under the conditions of this study, MA and 6MMPR depleted ATP equally. In addition, the toxicity of MA, alanosine, and 6MMPR were made as similar as possible. For example, the concentration of each agent used in the study resulted in a net decrease in cell density after 24 hr of treatment. Furthermore, cell death was first observed after approximately 8 hr of incubation with each of the compounds (defined as the detection of cells with less than a G1 complement of DNA by flow cytometry). Therefore, the measurements taken between 2 and 6 hr of treatment with each drug were on a population of intact cells under similarly toxic conditions.

The results of this study demonstrate that there are distinguishable differences between the biochemical effects of guanine nucleotide depletion and adenine nucleotide depletion. Nucleic acid synthesis, DNA synthesis in particular, appears to be a specific target of guanine nucleotide depletion. In contrast, adenine nucleotide depletion may cause a less specific cellular toxicity.

The biological consequences resulting from the inhibition of de novo purine biosynthesis are primarily mediated by the depletion of guanine nucleotides. The most conspicuous biochemical effect of purine starvation, the dramatic inhibition of DNA synthesis, is almost exclusively a consequence of guanine nucleotide depletion. DNA synthesis was drastically inhibited when guanine nucleotides were reduced to similar levels by inhibition of guanine nucleotide biosynthesis. However, selective depletion of adenine nucleotides to the levels caused by the inhibition of early de novo purine biosynthesis did not affect DNA synthesis. Also, in cells with inhibited early de novo purine biosynthesis, the selective return of adenine nucleotide levels to normal by adenine did not reestablish DNA synthesis. Only the selective return of guanine nucleotide levels to normal by guanine allowed DNA synthesis to continue. Similar results were observed in L5178Y cells treated with methotrexate (11).

The effects of purine starvation on the progression of cells through the cell cycle appear to be completely dependent upon the depletion of guanine nucleotides. Guanine nucleotide depletion and adenine nucleotide depletion had distinguishable effects on the progression of cells through the cell cycle (Chart 4). The effects of guanine nucleotide depletion and inhibition of early de novo purine biosynthesis on cell cycle progression were identical and highlight the similarity of their actions. The dramatically different profile caused by adenine nucleotide depletion is itself an interesting observation. Cells accumulate within early S phase at a time when DNA synthesis is affected minimally (Table 2), the rate of cell division remains normal (data not shown), and the cells appear to be progressing normally through late S, G2, and M to G1. In N1S1-67 cells, a quicker inhibition of cell division by alanosine within 2 hr was observed (7). Therefore, we had originally expected an accumulation of cells in G2-M. The difference between the 2 cell lines is not understood currently.

Adenine nucleotide depletion does not appear to contribute additional toxicity beyond that of guanine nucleotide depletion when early de novo purine biosynthesis is inhibited. In fact, the depletion of ATP can cause an acceleration of purine synthesis and thereby may antagonize the original de novo block (25). A decrease or an imbalance in levels of the deoxynucleotidetriphosphates has been thought to reflect the antipurine effects of methotrexate (12, 20, 21). Also, it has been suggested that the depletion of dGTP causes the DNA synthesis inhibition and toxicity of MA (15); however, in a previous study, we have demonstrated that the toxicity resulting from the inhibition of the de novo synthesis of guanine nucleotides in S-49 cells is related to the depletion of GTP rather than of dGTP (4). DNA synthesis inhibition does not result from insufficient deoxynucleotide substrate but from interruption of a process essential for DNA synthesis which uses either GTP or some nucleotide or cofactor, other than dGTP, which is derived from guanine ribonucleotides.

Therefore, we propose that the depletion of guanine ribonucleotides is primarily responsible for the toxicity resulting from the inhibition of early de novo purine biosynthesis. The possibility that the specific inhibition of de novo guanine nucleotide biosynthesis alone is more effective and/or selective than total purine starvation in chemotherapy must be studied. Therefore, we plan to investigate the interaction between guanine and adenine nucleotide depletion and its effect on cellular toxicity as well as to continue our study of the processes of DNA synthesis which may use GTP.

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REFERENCES

Toxicity of Purine Depletion

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