Consequences of Inhibition of Guanine Nucleotide Synthesis by Mycophenolic Acid and Virazole

Jeffrey K. Lowe, Larry Brox, and J. Frank Henderson

University of Alberta Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, Edmonton, Alberta, Canada T6G 2H7

SUMMARY

Mycophenolic acid and virazole are inhibitors of inosinate dehydrogenase and produce growth inhibition and loss of viability in cultured murine lymphoma L5178Y cells. Treatment with 1 μM mycophenolic acid produced the following changes in concentrations of acid-soluble nucleotides: (a) guanosine triphosphate decreased to less than 10% of control within 2 hr; (b) uridine triphosphate and cytidine triphosphate concentrations increased markedly; (c) adenosine triphosphate did not change; (d) deoxyguanosine triphosphate decreased; and (e) thymidine triphosphate increased. DNA synthesis was inhibited by 90% within 2 hr, whereas the incorporation of adenosine into RNA and of leucine into protein were much less affected. Virazole (100 μM) produces similar effects. These biochemical effects of mycophenolic acid, as well as its effects on cell growth, can be prevented by addition of guanylate to the medium. Mycophenolic acid treatment also appears to cause breakdown of high-molecular-weight DNA.

INTRODUCTION

Although mycophenolic acid has been known for more than 80 years, its ability to inhibit the growth of a variety of solid mouse and rat tumors has been demonstrated only within the last 10 years (20, 21, 23). Franklin and Cook (2) showed that mycophenolic acid was a potent inhibitor of nucleic acid synthesis in mammalian cells, and this was due to inhibition of the enzyme inosinate dehydrogenase (IMP-NAD oxidoreductase, EC 1.2.1.14). The structure of mycophenolic acid is shown in Chart 1, and the point in the network of reactions of purine metabolism at which it acts is shown in Chart 2.

Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximide) also inhibits inosinate dehydrogenase (19). Its structure is also shown in Chart 1.

Inosinate dehydrogenase catalyzes the conversion of inosinate to xanthylate, and inhibition of this reaction would be expected to result in a decrease in the synthesis and concentration of guanine nucleotides in animal cells grown in culture in media devoid of guanine. Guanine nucleotides are required as substrates or activators in a number of reactions of cellular metabolism, including DNA, RNA, and protein biosynthesis; adenylosuccinate synthetase (EC 6.3.4.4); ribonucleoside diphosphate reductase (EC 1.17.4.1); succinate thiokinase (EC 6.2.1.4); phosphoenolpyruvate carboxykinase (EC 4.1.1.32); CTP synthetase (EC 6.3.4.2); and microtubule polymerization.

In an attempt to understand better how inhibition of inosinate dehydrogenase leads to growth inhibition and loss of cell viability, we have measured the changes in guanine nucleotide concentrations that result from treatment with mycophenolic acid and the consequences of these changes for certain aspects of cellular metabolism. Some studies were also conducted using virazole.

MATERIALS AND METHODS

Purine and pyrimidine bases, nucleosides, and nucleotides were supplied by Sigma Chemical Co., St. Louis, Mo. Sucrose (density gradient grade) was supplied by Schwartz/Mann, Orangeburg, N. Y. Mycophenolic acid and virazole were the generous gifts of Dr. T. J. Franklin and Dr. R. K. Robins, respectively.

The murine lymphoma L5178Y cells used in this study were grown in stationary suspension culture in Fischer's medium supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, N. Y.). Stock cultures were maintained in logarithmic phase by dilution at least every 48 hr to a density of ca. 0.2 × 10^6 cells/ml. In the experiments reported here, cells were used at densities between 0.2 and 2.5 × 10^6 cells/ml, under which conditions the average generation time was 11 hr. Cell densities were measured using a Model Z, Coulter counter.

Cell viability was measured using a modification of the semisolid agar cloning method of Puck and Marcus (12). Cells were introduced into 100-× 17-mm plastic culture tubes (Miles Laboratory Inc., Naperville, Ill.) in 3 ml of Fischer's medium containing 10% horse serum, 10% 5-day conditioned medium, and 0.14% agar. The tubes were incubated at 37° for 7 to 10 days in a CO2 incubator, after which visible clones were counted. Untreated lymphoma L5178Y cells had a cloning efficiency of ca. 60%.

The incorporation of radioactive precursors into acid-insoluble material was measured as described previously (3). [Methyl-3H]Thymidine (3 Ci/m mole), [8-3H]adenosine (14 Ci/m mole), [2-14C]cytidine (28 mCi/m mole), [6-3H]uridine (23 Ci/m mole), [2-14C]uridine (51 mCi/m mole), and L-[4,5-3H]leucine (15 Ci/m mole) were obtained from Schwartz/Mann. Cells (2 ml) were placed in 12- × 75-mm stopped tubes in a water bath at 37°. The appropriate
Chart 1. Structures of mycophenolic acid and virazole.

Chart 2. Pathways of purine nucleotide synthesis de novo and from guanine in cultured lymphoma L5178Y cells. Heavy arrow, the reaction that is catalyzed by inosinate dehydrogenase and inhibited by mycophenolic acid and virazole.

radioactive precursor (1 μCi/ml final concentration) was added to each of 4 tubes for each condition, and at 5-min intervals the contents of each tube were added to 10 ml of cold 0.4 M perchloric acid. After 3 washes with cold perchloric acid, the acid-insoluble material was dissolved in 1.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.), toluene-based scintillation fluid was added, and radioactivity measurements were made using a liquid scintillation counter.

Concentrations of acid-soluble purine and pyrimidine ribonucleotides were determined spectrophotometrically after their separation by high-pressure liquid chromatography using a Reeve Angel Partisil-10 SAX column (25 cm × 4.6 mm; Mandel Scientific, Montreal, Quebec, Canada) and a linear gradient of potassium phosphate and potassium chloride, pH 4.5, as described in detail elsewhere (17).

Concentrations of deoxyribonucleoside triphosphates were measured enzymatically, using a modification of the procedure originally developed by Solter and Handschumacher (18), as modified by Lowe and Grindley (7). In this modification, the DNA polymerase (EC 2.7.7.7) was obtained from Micrococcus luteus (P-L Biochemicals, Milwaukee, Wis.) and was used at a concentration of 0.3 unit/assay tube. MnCl₂ was used as the divalent cation, at a final concentration of 0.1 mM. The assay was linear within the range of 2 to 50 pmoles of each deoxyribonucleoside triphosphate.

Changes in DNA structure were detected by determining the molecular weight of DNA single strands by a modification of the alkaline sucrose density method of Walters and Hildebrand (22). Lymphoma L5178Y cells were grown with 1 pCi [³H]thymidine per ml for 2 to 3 hr, after which they were washed and resuspended in warm Fischer’s medium plus horse serum. These cells were allowed to grow for at least an additional 4 hr before drug treatment was initiated. After drug treatment as specified in “Results,” the cells were collected by centrifugation and resuspended in 0.05 M cacodylic acid (pH 7.0) at a density of 1 × 10⁶ cells/ml. In the meantime, tubes had been prepared that contained a 5 to 20% (w/v) gradient of sucrose dissolved in 0.4 M NaOH, 0.1 M sodium EDTA, and 0.1% sodium lauryl sarcosine on top of a cushion consisting of 5 ml of 50% sucrose. The lysing buffer, consisting of 0.4 ml of 1 mg heparin per ml and 1% sodium lauryl sarcosine, was layered on top of the alkaline sucrose gradient. Cell suspensions (100 μl) were layered carefully onto the lysing buffer, and, after 15 min at room temperature, 1 ml of 0.5 M NaOH plus 0.1 M EDTA were layered carefully on top of the cells. After an additional 45 min at room temperature, the tubes were centrifuged in a Beckman Model L65-B centrifuge at 22,000 rpm for 5 hr at 15°C, using an SW 27 head.

The gradients were fractionated by pumping 50% sucrose into the bottom of the tubes with an ISCO gradient extractor (Instrumentation Specialties Co., Lincoln, Nebr.). The 1.2-ml fractions were acidified with 2 N HCl and filtered onto Whatman GF/A filters, washed with more acid, and dried; their radioactivity was measured using a toluene-based scintillation fluid. In control samples, recovery of radioactivity applied to the gradient was ca. 90%.

DNA was determined spectrofluorimetrically by a modification of the procedure originally developed by LePelq and Paolletti (5), as modified by Morgan and Pulleyblank (9). Approximately 400,000 cells were collected by centrifugation for 45 sec in a Beckman Microfuge B centrifuge. The cell pellet was resuspended in 0.1 ml of 0.3 N NaOH and heated in a boiling water bath for 5 min. After cooling, 0.1- to 0.05-ml portions were added to 2 ml of ethidium bromide (0.5 μg/ml) in 5 mM Tris-HCl and 0.5 mM EDTA, pH 8.0, and the fluorescence was measured using a Turner Model 430 spectrofluorimeter. The excitation and emission wave-
lengths were 525 and 600 nm, respectively. Calf thymus DNA was used as a standard.

A volume distribution analysis of control and mycophenolic acid-treated L5178Y cells was performed using a Coulter Model Z, counter equipped with the 100-channel Coulter Channelizer II. The output from the pulse height analyzer was connected to an X-Y recorder. The instrument was calibrated with polystyrene beads having diameters of 9.53 and 18.04 µm (Coulter Electronics, Hialeah, Fla.).

The relative DNA content of the mycophenolic acid-treated L5178Y cells was determined by microfluorimetry after propidium iodide staining. Cells were collected by centrifugation and stained for 15 min with propidium iodide (0.05 mg/ml) in 0.1% sodium citrate at a density of 400,000 cells/ml (4). The cellular fluorescence intensities were recorded using a Bio/physics Model 4800 A cytofluorograph equipped with a Model 2100 pulse height analyzer (Bio/physics Systems, Inc., Mahopac, N. Y.). The cell count in each of the 100 channels was printed on a teletype.

RESULTS

To relate biochemical changes resulting from mycophenolic acid action to the biological effects of the drug, it was necessary first to determine drug concentrations that were growth inhibitory. Chart 3 shows that continuous exposure to 1 µM mycophenolic acid resulted in less than 1 doubling in cell number of cultured lymphoma L5178Y cells. A concentration of 0.32 µM mycophenolic acid caused less severe growth inhibition, and 0.1 µM drug had no effect on the growth of these cells.

Growth inhibition by virazole is demonstrated in Chart 4. On a molar basis, about 100 times more virazole than mycophenolic acid is required to achieve an equivalent degree of growth inhibition.

To relate the concentration of mycophenolic acid that was found to be growth inhibitory to its known biochemical effect, the apparent activity of inosinate dehydrogenase was measured in lymphoma L5178Y cells exposed to 1 µM mycophenolic acid. This was done by measuring the extent to which the conversion of [14C]hypoxanthine to xanthylate plus guanine nucleotides was inhibited in the presence of drug (16). Following exposure of cells to 1 µM mycophenolic acid for 30 min, the apparent activity of inosinate dehydrogenase was reduced to between 20 and 40% of control in several replicate experiments. This is similar to the effect of this concentration of drug in Ehrlich ascites tumor cells both in vitro (14) and in vivo (15). No greater inhibition of inosinate dehydrogenase resulted when the concentration of mycophenolic acid was increased to as much as 32 µM. The extent of inhibition of inosinate dehydrogenase produced by virazole in this system was not studied.

If inosinate dehydrogenase is the sole direct site of action of mycophenolic acid in growing lymphoma L5178Y cells, it should be possible to prevent its growth-inhibitory effect by supplying guanine exogenously to permit guanine nucleotide synthesis via hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8). Indeed, when lymphoma L5178Y cells were grown with both 1 µM mycophenolic acid and 100 µM guanylate, no inhibition of growth was apparent. Guan-

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Footnote: Only partial reversal of growth inhibition by mycophenolic acid was achieved when either guanine or guanosine was used in place of guanylate. This inactivity may have resulted from rapid cleavage of guanosine to guanine and rapid deamination of guanine; horse serum is known to have a high guanine deaminase activity (17). The use of guanylate as a source of intracellular guanine compounds was based on the recent study of Sakaguchi et al. (13). Although the metabolism of the exogenously supplied guanylate was not studied, it seems likely that it was slowly converted to guanine by dephosphorylation and phosphorolysis; the low concentrations of guanine that presumably result could be used preferentially for nucleotide formation. In addition, or alternatively, nucleoside monophosphates can be used directly, at least to some extent (11).
ylate at this concentration also produced substantial reversal of the growth-inhibitory effects of 100 μM virazole.

Further experiments showed that not only did mycophenolic acid and virazole inhibit growth, but also treatment with either drug led to loss of cell viability. The survival, in terms of ability to form colonies in soft agar, of lymphoma L5178Y cells exposed to mycophenolic acid is shown in Chart 5. A drug concentration of 0.32 μM was not highly lethal, whereas 1 μM mycophenolic acid caused about 2 logs of cell kill after treatment for 24 hr. Inclusion of 100 μM guanylate in the cloning medium did not affect the survival of mycophenolic acid-treated cells, nor did it change the plating efficiency of untreated cells. Chart 5 also shows the results of 1 experiment in which the effect of 100 μM virazole on the survival characteristics of lymphoma L5178Y cells was measured. This drug concentration, which produced about the same degree of growth inhibition as 1 μM mycophenolic acid, also produced approximately the same effects on cell viability as 1 μM mycophenolic acid.

In all further studies of secondary biochemical effects, 1 μM mycophenolic acid and 100 μM virazole were used in order to be able to relate biochemical changes to known degrees of growth inhibition and loss of cell viability.

Because mycophenolic acid reduces the rate of guanine nucleotide synthesis de novo, we next studied the changes in intracellular concentrations of purine and pyrimidine ribonucleotides that resulted from treatment with this drug. Chart 6 reports concentrations of ribonucleoside triphosphates and shows that rapid and marked changes were caused by mycophenolic acid. Thus the concentration of GTP decreased to less than 10% of control values after about 2 hr of treatment, whereas the concentrations of UTP and CTP first increased to about 2.5 times control levels and then declined. The concentration of ATP did not change much during the course of a 12-hr treatment with mycophenolic acid. These data agree with results of similar experiments using leukemia L1210 cells, which have recently been reported by Nelson et al. (10).

For further information regarding the effects of mycophenolic acid on nucleotide metabolism, intracellular concentrations of purine and pyrimidine deoxyribonucleoside triphosphates were measured. Changes in these concentrations in lymphoma L5178Y cells treated with mycophenolic acid and virazole are shown in Charts 7 and 8, respectively. In Chart 7, it can be seen that mycophenolic acid treatment caused a large increase in the concentration of TTP; the concentration of this nucleotide reached a plateau at about 250% of control value after about 2 hr of drug treatment. In contrast, the concentration of dGTP decreased to about 30% of control, with the most rapid decrease occurring in the 1st hr. A smaller decrease in the concentration of dCTP was observed, whereas the concentration of ATP changed only slightly.

Chart 8 shows that the changes in deoxyribonucleoside triphosphate concentrations produced by treatment with virazole were similar to those caused by mycophenolic acid. It was noted above that addition of a source of preformed guanine (i.e., guanylate) prevented the antiproliferative effects of mycophenolic acid. It thus seemed appropriate to determine the effects of guanylate on nucleotide concentra-
Reversal of some of the biochemical effects of mycophenolic acid by 100 pM guanylate

For ribonucleoside triphosphate concentrations, the cells were incubated for 3 hr, whereas, for deoxyribonucleoside triphosphate concentrations, the incubation period was 4.75 hr. In both sets of experiments, the guanylate and mycophenolic acid were added simultaneously. All numbers represent determinations from a single sample.

<table>
<thead>
<tr>
<th>Concentrations (nmoles/10^9 cells)</th>
<th>ATP</th>
<th>CTP</th>
<th>UTP</th>
<th>GTP</th>
<th>dATP</th>
<th>dCTP</th>
<th>TTP</th>
<th>dGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2128</td>
<td>288</td>
<td>584</td>
<td>411</td>
<td>11</td>
<td>53</td>
<td>36</td>
<td>13</td>
</tr>
<tr>
<td>1 µM mycophenolic acid</td>
<td>1667</td>
<td>479</td>
<td>997</td>
<td>92</td>
<td>9</td>
<td>39</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>1 µM mycophenolic acid + 100 µM GMP</td>
<td>2111</td>
<td>303</td>
<td>511</td>
<td>833</td>
<td>10</td>
<td>32</td>
<td>21</td>
<td>14</td>
</tr>
</tbody>
</table>

1% of control after 3 hr of treatment. This potent inhibition was completely prevented by addition of 100 µM guanylate. Virazole produced very similar inhibition of DNA synthesis (data not shown).

As a consequence of the inhibition of DNA synthesis produced by mycophenolic acid, it might be expected that cells would not proceed through the cell cycle but would accumulate in G1, or at the beginning of S phase. Chart 10 shows an initial decrease in the G2 peak as a function of time of treatment with 1 µM mycophenolic acid. There is also a simultaneous accumulation of cells in early S phase, indicated by the pronounced shoulder in the 9-hr cytogram.

In further experiments, the effects of mycophenolic acid on RNA and protein synthesis were determined. When radioactive adenosine and cytidine were used as indicators of RNA synthesis, mycophenolic acid had much less effect than it had on DNA synthesis (Table 2). Thus after correction for the small changes in ATP concentrations, the rate of
These incorporation studies were carried out as described in the text. The cells were incubated for the indicated period in the presence of 1 μM mycophenolic acid before the addition of the appropriate precursor. The correction for incorporation of counts into acid-insoluble material was made by multiplying the incorporation rate (expressed as a percentage of control) by the pool size of the ultimate precursor into nucleic acid (expressed as a fraction of control).

Incorporation (corrected for treatment)

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Length of treatment (hr)</th>
<th>Incorporation (% of control)</th>
<th>Incorporation (corrected for pool changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[8-3H]Adenosine</td>
<td>0.5</td>
<td>64</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>70</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>72</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td>[2-14C]Cytidine</td>
<td>0.5</td>
<td>44</td>
<td>62</td>
</tr>
<tr>
<td>L-[3,4-3H]Leucine</td>
<td>1</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

Chart 9. 3[H]Thymidine incorporation into acid-insoluble material of L5178Y cells exposed to 1 μM mycophenolic acid for 0 hr (●), 0.5 hr (○), or 2 hr (■), as described in the text.

Chart 10. Effect of mycophenolic acid on the cell cycle. DNA frequency histogram of L5178Y cells after exposure to 1 μM mycophenolic acid for the indicated times. In each case 50,000 cells were counted.

incorporation of adenosine into acid-insoluble material was reduced to 50% of control only after 4 hr of drug treatment. Even after 12 hr, when about 90% of the cells cannot form colonies, the incorporation of both adenosine and cytidine remained at about 40% of control (data not shown). Table 2 also shows that incorporation of radioactive leucine into acid-insoluble material was reduced only by 35% even after 6 hr of treatment with mycophenolic acid.

As expected, a consequence of continued RNA and protein synthesis (even though reduced partially) in the presence of virtually complete inhibition of DNA synthesis is "unbalanced growth" and hence an increase in the size of the cells. L5178Y cells underwent a gradual increase in cell volume during mycophenolic acid treatment. The ratio of modal cell volume for treated to control cells for various times was: 3 hr, 1.0; 6 hr, 1.14; 9 hr, 1.22; and 17 hr, 1.45. At the longer time periods, the size distribution for treated cells became skewed towards larger cell volumes.

The studies described provide a reasonable basis for understanding the growth-inhibitory effects of mycophenolic acid and virazole. However, these data by themselves do not appear to be sufficient to explain the loss of cell viability that is produced by treatment with these 2 drugs. The possibility that changes in DNA structure might be produced in drug-treated cells has been considered, and initial attempts have been made to study this matter.

The total amount of DNA in control and mycophenolic acid-treated cells, as determined by a sensitive spectrofluorimetric method, was 11.8 and 10.5 pg/cell, respectively. Thus no marked amount of DNA was lost from treated cells; the small change that was observed might be explained by the observed effect of mycophenolic acid on the cell cycle.

Next, gross breakdown of DNA was studied by measuring the loss of radioactivity into the medium with control and treated cells the DNA of which had been labeled by prior incubation with [3H]thymidine. It was found that 1 μM mycophenolic acid in fact did not increase the rate of loss of radioactivity (either acid soluble or acid insoluble) from the cells at times up to 22 hr of treatment.

In subsequent experiments the effect of mycophenolic acid on the molecular weight of DNA single strands was determined by alkaline sucrose density centrifugation. Chart 11 shows that mycophenolic acid treatment leads to a time-dependent loss of high-molecular-weight DNA in lymphoma L5178Y cells. This is indicated by the decrease in amount of radioactivity in the high-molecular-weight fraction, as the same total amount of radioactivity was used in all cases and no radioactivity was found either in the sucrose cushion or at the bottom of the tubes. However,
these functions cannot be ruled out. GTP is also required that guanylate reverses the growth inhibition produced by the drug. The concentration of mycophenolic acid used in the biochemical studies allowed less than a doubling of the initial cell number and caused an exponential loss of viability as determined by alkaline sucrose gradient centrifugation.

DISCUSSION

In these studies we have attempted to delineate the biochemical and biological consequences of inhibition of inosinate dehydrogenase in cultured lymphoma L5178Y cells. The concentration of mycophenolic acid used in the biochemical studies allowed less than a doubling of the initial cell number and caused an exponential loss of viability as measured by the semisolid agar cloning method. The most direct relationship observed is between loss of cell viability and degradation of DNA, as determined by alkaline sucrose gradient centrifugation.

Chart 12 shows a plot of a number of biochemical parameters of mycophenolic acid action versus the loss of cell viability produced by the drug. The most direct relationship observed is between loss of cell viability and degradation of DNA, as determined by alkaline sucrose gradient centrifugation.

Chart 12. Plot of biochemical parameters of mycophenolic acid action versus the loss of cell viability produced by this drug. All data are presented as a percentage of control. A, [methyl-3H]thymidine incorporation; Δ, GTP concentration; ○, dGTP concentration; ○, [5-bromo-2′-deoxyadenosine (AR) incorporation; ●, dTTP concentration; ■, size of the full-length DNA peak after alkaline sucrose density gradient centrifugation.

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fragments of DNA of intermediate molecular weight were not detected.

The observed elevations in pyrimidine ribonucleotide concentrations following mycophenolic acid treatment are consistent with the inhibition of pyrimidine nucleotide metabolism. The concentrations of pyrimidine nucleotides increase in cells in which guanine ribonucleotide concentrations are lowered (J. Barankiewicz and J. F. Henderson, unpublished observation) and that elevated guanine ribonucleotide concentrations inhibit pyrimidine metabolism has not yet been studied in detail in mycophenolic acid-treated lymphoma L5178Y cells, the observation that concentrations of both UTP and CTP are elevated to the same extent following mycophenolic acid treatment suggests that GTP concentrations still are not limiting for the CTP synthetase reaction.

Decreased concentrations of guanine ribonucleotides appear to lead directly to decreased synthesis of guanine deoxyribonucleotides, whereas elevated concentrations of dUTP appear to lead to increased rates of dTTP synthesis. Why dCTP concentrations are somewhat reduced while CTP concentrations are elevated too slow and stop cycling. It is surprising that the cells do not simply accumulate at the G1-S interface, based on the usual model of allosteric feedback regulation of ribonucleotide reductase.

As a further step in this chain of events, DNA synthesis appears to be severely inhibited as a consequence of reduced dGTP concentration. In addition, however, the possibility must be considered that the observed imbalance in concentrations of the 4 deoxynucleoside triphosphates also contributes to this inhibition of DNA synthesis.

Data obtained by cytofluorography suggest that the cells that are in S phase at time of addition of mycophenolic acid slow and stop in S phase, whereas all other cells continue around the cell cycle until they enter S phase, where they too slow and stop cycling. It is surprising that the cells do not simply accumulate at the G1-S interface, based on the measured, very low DNA-synthetic rate remaining after 1 to 2 hr of treatment with 1 μM mycophenolic acid. The observed increase in cell size is expected of cells in which

The observed elevation in pyrimidine ribonucleotide concentrations following mycophenolic acid treatment may result in part from accelerated de novo synthesis of pyrimidines consequent upon accelerated synthesis of PP-ribose-P. This suggestion is based on observations in another cell system that PP-ribose-P accumulation increases in cells in which guanine ribonucleotide concentrations are lowered (J. Barankiewicz and J. F. Henderson, unpublished observation) and that elevated guanine ribonucleotide concentrations inhibit PP-ribose-P synthesis (1). Although pyrimidine metabolism has not yet been studied in detail in mycophenolic acid-treated lymphoma L5178Y cells, the observation that concentrations of both UTP and CTP are elevated to the same extent following mycophenolic acid treatment suggests that GTP concentrations still are not limiting for the CTP synthetase reaction.

• The abbreviation used is: PP-ribose- P, phosphoribosylpyrophosphate.
RNA and protein synthesis continue (although at a reduced rate) while DNA synthesis is nearly totally inhibited.

The consequences of inhibition of inosinate dehydrogenase thus far discussed are sufficient to explain the growth-inhibitory effects of mycophenolic acid and virazole. However, growth inhibition is followed by loss of cell viability, and this is more difficult to explain in biochemical terms; in fact little is known about biochemical bases of cell death in general. Chart 12 shows that there is no simple relationship between most of the biochemical parameters measured and loss of cell viability.

Experiments using alkaline sucrose gradient centrifugation of DNA from cells previously labeled with $[^3H]$thymidine have provided evidence that breaks are produced which accumulate in the treated with mycophenolic acid. Presumably, these breaks either are induced by the lowered dGTP concentrations (or by the overall imbalance in deoxyribonucleoside triphosphate concentrations) or are produced in the normal course of cell growth and cannot be repaired in mycophenolic acid-treated cells. Although these observations must be pursued in greater detail and the possibility must be considered that they are a result of cell death rather than a cause of it, they remain a promising lead.

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