Comparison of the Effects of Several Inhibitors of the Synthesis of Nucleic Acids upon the Viability and Progression through the Cell Cycle of Cultured H. Ep. No. 2 Cells

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SUMMARY

The effects of arabinosylcytosine, hydroxyurea (HU), guanazole (Gz), 5-fluorodeoxyuridine, methotrexate, 6-mercaptopurine, 6-methylthiopyrimidine ribonucleoside, 6-thioguanine, and 5-fluorouracil (5-FUra) upon the viability and progression through the cell cycle of cultured H. Ep. No. 2 cells have been determined. Similarities and differences were found.

None of the agents prevented the cells that were initially in G$_2$ from progressing to mitosis. All of the agents retarded or prevented progression to mitosis of cells initially in S but, by the end of a period of exposure equal to $T_C$, essentially all of the cells initially in S and exposed to 6-mercaptopurine, 6-methylthiopyrimidine ribonucleoside, and the lower concentrations of 5-FUra reached mitosis.

When the logarithm of the surviving fraction after an exposure period equal to $T_C$ was plotted versus concentration of the agent, only 5-FUra yielded an exponential curve. With all of the other agents, the curves flattened for higher concentrations, which shows that increasing the concentrations of these agents beyond certain levels does not increase cell kill during a 26-hr exposure period. Thus, only 5-FUra could be expected to cause "total cell kill" under these conditions.

None of the agents prevented the cells that were initially in G$_1$ from progressing to mitosis. All of the agents retarded or prevented progression to mitosis of cells initially in S but, by the end of a period of exposure equal to $T_C$, essentially all of the cells initially in S and exposed to 6-mercaptopurine, 6-methylthiopyrimidine ribonucleoside, and the lower concentrations of 5-FUra reached mitosis.

All of the agents prevented cells initially in G$_1$ from progressing to mitosis during an exposure period equal to $T_C$.

Arabinosylcytosine, HU, Gz, methotrexate, 5-fluorodeoxyuridine, and 5-FUra caused partial synchronization of the cultures at or near the G$_1$-S transition stage. (This blocking effect might protect some of the cells from the agents by preventing them from progressing to a more sensitive stage of the cycle.) There was no evidence that 6-mercaptopurine, 6-methylthiopyrimidine ribonucleoside, and 6-thioguanine caused synchronization.

It is probable that the concentrations of HU and Gz decreased during the 26-hr incubation with H. Ep. No. 2 cells.

It appears that arabinosylcytosine, HU, and Gz are more effectively removed from the cells by washing than are the other agents.

INTRODUCTION

Several anticancer agents are known to interfere with the synthesis of nucleic acids by different means, as indicated in Table 1. The effects of some of these agents upon the viability and progression through the cell cycle of cultured cells have been studied by several investigators. However, for several other agents, such investigations have not been performed. Therefore, it seemed worthwhile to compare the effects of all of these agents in a single set of experiments. It is possible that the data obtained in these experiments will be useful in improving the methods of using the individual agents and in selecting combinations of these agents that will yield additive or synergistic therapeutic effects.

MATERIALS AND METHODS

Cells. H. Ep. No. 2 cells (42) were grown on SRI-14 medium (29) by methods that have been described (54), with the modifications designated below in the procedures. The cell cycle of these cells under these conditions has been analyzed (54), and the lengths of the phases and the distribution of cells of an exponentially growing, nonsynchronized culture among these phases are given under "Results."

Sources of Compounds. Thymidine-methyl-3H, with a specific activity of 6.7 Ci/m mole, was purchased from New England Nuclear, Boston, Mass., and crystalline Colcemid was obtained from Ciba Pharmaceutical Company, Summit, N. J. The sources of the inhibitors were as follows: HU, Mann Research Laboratories, New York, N. Y.; Meth, American Cyanamid Company, Pearl River, N. Y.; 6-MP, Burroughs Wellcome and Company, Tuckahoe, N. Y.; 6-TG, 5-FUra, 5-FdUrd, ara-C, Gz, and 6-MeTPR, Drug Research and Development, Chemotherapy, National Cancer Institute, NIH.

Determination of Cytotoxicity. One hundred thousand cells were plated into each of a number of 1-oz prescription bottles with 5 ml of medium and were incubated at 37°. At 24 hr, the medium was decanted, and fresh medium was added. At 48 hr, the medium was decanted again, fresh medium containing designated concentrations of specified agents was added, and incubation was continued for 26 hr. [Previous experiments...
Cultures were set up in 1-oz bottles and refed at 24 hr, as described above and were refed at 24 hr. At 48 hr, the cultures were determined. The plating efficiency for the control cultures at the end of the 26-hr incubation period in normal medium. Then, the cell count in each culture at the beginning of the 26-hr period should be $5 \times 10^5$ cells. Since preliminary results obtained in this laboratory have shown that a variety of agents do not interfere with the progression of cells initially in G2 to mitosis and division, the long-and-short-dash line in Chart 1 is drawn to show the cell count that would be obtained if the initial count were $5 \times 10^5$ and if only the cells initially in G2 and M proceeded to divide during the 26-hr period of incubation. All of the agents at the lower concentrations limited the extent of multiplication of the cells, and it is unlikely that many cells other than those initially in G2 or M proceeded to divide during the period of exposure to the agents, and in some instances it appears that the cells initially in G2 and M did not divide.

The fractions of the populations of cells that survived in cultures exposed to the various concentrations of the agents at the end of the 26-hr exposure period were determined by measurement of the ability of the washed cells to produce clones during incubation in normal medium. Chart 2 shows that only 5-FUra gave an exponential curve when the logarithm of the surviving fraction was plotted against the concentration of the agent. With the other agents, the curves flattened for the higher concentrations of the agents. Knowledge of these parameters is helpful in interpreting some of the data obtained in this study and presented in this report.

**RESULTS**

**Kinetic Parameters.** The kinetic parameters of the cell cycle of H. Ep. No. 2 cells that were grown in glass-attached cultures in SRI-14 medium are as follows: $T_C$, 26.1 hr; $T_G1$, 14.4 hr; $T_S$, 6.9 hr; $T_G2$, 3.9 hr; and $T_M$, 0.9 hr (54). For an asynchronous, exponentially growing culture of these cells, the calculated distribution of the cells among the various phases is 63.6% in G1, 22.8% in S, 11.1% in G2, and 2.5% in M. Knowledge of these parameters is helpful in interpreting some of the data obtained in this study and presented in this report.

Effects on Proliferation and Clonogenicity. Chart 1 shows the effects of increasing concentrations of the agents upon the multiplication of cells during a 26-hr exposure of glass-attached cells to SRI-14 medium containing the agents. The values are normalized to a cell count of $10^6$ cells for the control cultures at the end of the 26-hr incubation period in normal medium. Then, the cell count in each culture at the beginning of the 26-hr period should be $5 \times 10^5$ cells. Since preliminary results obtained in this laboratory have shown that a variety of agents do not interfere with the progression of cells initially in G2 to mitosis and division, the long-and-short-dash line in Chart 1 is drawn to show the cell count that would be obtained if the initial count were $5 \times 10^5$ and if only the cells initially in G2 and M proceeded to divide during the 26-hr period of incubation. All of the agents at the lower concentrations limited the extent of multiplication of the cells, and it is unlikely that many cells other than those initially in G2 or M proceeded to divide during the period of exposure to the agents, and in some instances it appears that the cells initially in G2 and M did not divide.

The fractions of the populations of cells that survived in cultures exposed to the various concentrations of the agents at the end of the 26-hr exposure period were determined by measurement of the ability of the washed cells to produce clones during incubation in normal medium. Chart 2 shows that only 5-FUra gave an exponential curve when the logarithm of the surviving fraction was plotted against the concentration of the agent. With the other agents, the curves flattened for the higher concentrations of the agents.

Effects upon Progression through the Cell Cycle during Exposure to the Agents. In these experiments, the Colcemid block procedure was used, and the concentrations of the agents (with the exception of 5-FUra) corresponded to points of medium containing designated concentrations of the agents, and incubation in the presence of the agents was continued for 26 hr. At the end of this period, the medium was decanted, and the cells were rinsed in situ 2 times with SRI-14 salt solution and were refed with medium containing thymidine-methyl-3H, with or without Colcemid. At various times thereafter, cells were harvested and used for the preparation of slides for radioautography and classification. If Colcemid was present, it was possible to determine the rate and extent of accumulation of cells at mitosis; if Colcemid was absent, it was possible to determine the presence or absence of waves of mitoses. Rates of labeling in the presence or absence of Colcemid also yielded evidence of the degree of synchronization.

### Table 1

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode(s) of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C</td>
<td>Inhibition of DNA polymerase (21, 24, 40), incorporation into DNA (24, 40), and chain termination in DNA (53)</td>
</tr>
<tr>
<td>HU</td>
<td>Inhibition of reduction of ribonucleotides (7, 19, 35, 43)</td>
</tr>
<tr>
<td>Gz</td>
<td>Inhibition of reduction of ribonucleotides (11)</td>
</tr>
<tr>
<td>Meth</td>
<td>Inhibition of synthesis of purine ribonucleotides and thymidylate (5)</td>
</tr>
<tr>
<td>5-FdUrd</td>
<td>Inhibition of synthesis of thymidylate (26, 27)</td>
</tr>
<tr>
<td>5-FUra</td>
<td>Incorporation into RNA and inhibition of synthesis of thymidylate (26, 27)</td>
</tr>
<tr>
<td>6-MP</td>
<td>Inhibition of de novo synthesis of IMP and inhibition of interconversion of purine ribonucleotides (20, 41)</td>
</tr>
<tr>
<td>6-TG</td>
<td>Inhibition of de novo synthesis of IMP, inhibition of conversion of IMP to XMP, inhibition of GMP kinase, and incorporation into RNA and DNA (36, 41)</td>
</tr>
<tr>
<td>6-MeTPR</td>
<td>Inhibition of de novo synthesis of IMP (41)</td>
</tr>
</tbody>
</table>
that were well into the flattened portions of the growth-response curves (Chart 2). These concentrations were chosen in order that the maximum effects of the agents might be evident. Portions of the data are presented in Table 2. Although data were obtained at a number of times other than those designated in the headings of Table 2, the data presented are sufficient to justify the interpretations and conclusions given below.

The rate of accumulation of cells at metaphase in the presence of each of the agents was similar to that of the control culture for the 1st 4 hr, which indicates that none of the agents significantly interfered with the progression to mitosis of cells initially in G_2 (Table 2, Column 2). By 12 hr, the cells initially in G_2 and S should have arrived at mitosis, but only 6-MP, 6-MeTPR, and 5-FUra permitted cells initially in S to progress to mitosis during the interval between 4 and 12 hr (Table 2, Column 3), and these progressed at reduced rates. By 28 hr, essentially all of the cells initially in S in the

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Chart 1. Numbers of cells present in cultures following 26 hr of exposure to various concentrations of the agents. All values are normalized to a control culture of 1 x 10^6 cells/culture. - - , number of cells that would be present if there were 5 x 10^6 cells present initially and if only those in G_2 and M proceeded to divide. •, ara-C; X, Gz; O, HU; @, Meth; Δ, 6-MP; α, 6-TG; β, 6-MeTPR; γ, 5-FUra; ♡, 5-FdUrd. Scale A is applicable for 6-MP, 6-TG, 6-MeTPR, Meth, 5-FUra, and 5-FdUrd; Scale B is applicable for ara-C; and Scale C is applicable for HU and Gz.

Chart 2. Survival of cells (as determined by formation of clones) after exposure of cells to agents for 26 hr. See Chart 1, legend, for key to the curves and the use of the scales on the abscissa.

Table 2
Effects of agents upon the progression of H. Ep. No. 2 cells through the cell cycle during incubation in medium containing the agent, thymidine-methyl-3H, and colcemid

<table>
<thead>
<tr>
<th>Agent</th>
<th>% in mitosis at 4 hr</th>
<th>% labeled during 1st hr</th>
<th>% labeled at 12 hr</th>
<th>% labeled at 28 hr</th>
<th>% labeled mitoses at 28 hr</th>
<th>% labeled interphase cells at 28 hr</th>
<th>Surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>35</td>
<td>93</td>
<td>20</td>
<td>65</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>ara-C (50 μg/ml)</td>
<td>8</td>
<td>11</td>
<td>13</td>
<td>13</td>
<td>32</td>
<td>66</td>
<td>1</td>
</tr>
<tr>
<td>HU (50 μg/ml)</td>
<td>7</td>
<td>9</td>
<td>15</td>
<td>9</td>
<td>65</td>
<td>84</td>
<td>4</td>
</tr>
<tr>
<td>Gz (500 μg/ml)</td>
<td>7</td>
<td>10</td>
<td>16</td>
<td>12</td>
<td>67</td>
<td>83</td>
<td>2</td>
</tr>
<tr>
<td>5-FdUrd (10 μg/ml)</td>
<td>10</td>
<td>11</td>
<td>13</td>
<td>20</td>
<td>71</td>
<td>82</td>
<td>5</td>
</tr>
<tr>
<td>Meth (10 μg/ml)</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>24</td>
<td>59</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td>6-MP (10 μg/ml)</td>
<td>11</td>
<td>18</td>
<td>31</td>
<td>21</td>
<td>21</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>6-MeTPR (10 μg/ml)</td>
<td>7</td>
<td>19</td>
<td>35</td>
<td>19</td>
<td>38</td>
<td>56</td>
<td>20</td>
</tr>
<tr>
<td>6-TG (10 μg/ml)</td>
<td>7</td>
<td>11</td>
<td>13</td>
<td>18</td>
<td>30</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>5-FUra 1 μg/ml</td>
<td>10</td>
<td>28</td>
<td>56</td>
<td>21</td>
<td>74</td>
<td>83</td>
<td>43</td>
</tr>
<tr>
<td>3 μg/ml</td>
<td>11</td>
<td>21</td>
<td>28</td>
<td>22</td>
<td>72</td>
<td>78</td>
<td>15</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>79</td>
<td>73</td>
<td>7</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>8</td>
<td>17</td>
<td>18</td>
<td>21</td>
<td>70</td>
<td>73</td>
<td>7</td>
</tr>
<tr>
<td>15 μg/ml</td>
<td>11</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>75</td>
<td>8</td>
<td>67</td>
</tr>
</tbody>
</table>

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cultures treated with 6-MP, 6-MeTPR, and the lower concentrations of 5-FUra had reached mitosis; this did not occur with the other agents (Table 2, Column 4). All of the agents, except 5-FUra at the lowest concentrations, prevented cells initially in G1 from reaching mitosis during 28 hr.

The data in Table 2, Column 5, show the effects of the agents upon the labeling of cells during the 1st hr of exposure to medium containing the agent, thymidine-methyl-3H, and Colcemid. (This would supposedly show the effects of the agents upon the labeling of cells in S at the time the agent and the thymidine-methyl-3H were added.) 5-FUra, 5-FdUrd, and Meth did not prevent the labeling of cells initially in the S phase, since thymidine-methyl-3H would enter the metabolic pathway at a point subsequent to the block of thymidylate synthesis caused by these agents. 6-MP, 6-MeTPR, and 6-TG did not prevent the labeling of cells initially in S, since these agents do not directly interfere with the utilization of thymidine-methyl-3H and since the pools of purine ribonucleotides or deoxyribonucleotides within the cells initially were evidently great enough (2, 50) to permit enough synthesis of DNA to occur for the nuclei to become labeled. On the other hand, ara-C, HU, and Gz reduced the 1-hr pulse-label index from 20 down to 9 to 13. It is not known in which part(s) of the S phase the cells did not become labeled.

The data in Table 2, Columns 6 and 7, show the effects of the agents upon the progression of cells into S to the extent of taking up enough thymidine-methyl-3H to be scored as labeled. HU, Gz, 5-FdUrd, Meth, and 5-FUra had little or no effect upon the progression of cells from G1 into S far enough for the nuclei to become labeled during the 1st 12 hr; Meth inhibited progression between 12 and 28 hr. ara-C, 6-MP, 6-MeTPR, and 6-TG retarded the progression of cells from G1 into S throughout the 28-hr period. Although all of the agents permitted some of the cells initially in G1 to proceed into S, the extent of the synthesis of DNA by these cells is not known. Perhaps all of the agents prevented the cells initially in G1 from proceeding to mitosis during 28 hr (Table 2, Columns 8 and 9).

**Effects of 26-Hr of Exposure to the Agent upon Subsequent Progression of the Cells through the Cycle.** Only after treatment with ara-C, HU, or Gz did more than about 5% of the cells proceed to mitosis during the 26-hr postexposure examination period (Chart 3). This statement is applicable to 6-MP and 6-MeTPR, although the curves are not shown. Many of the cells that were exposed to 6-TG disintegrated during the 26-hr period after they were washed, and therefore the mitotic index had little significance. Since, with all of the agents, essentially all of the cells that did reach mitosis during this period were labeled, there were evidently few if any cells in G2 and M at the time that the cells were washed and initially placed in contact with the medium containing thymidine-methyl-3H and Colcemid.

Table 3 brings together some of the data of Table 2 and Charts 2 and 3. It appears that the effects of HU and Gz are partially reversible and that cell multiplication can continue to some extent after the cells are washed. Other data (see below) indicate that these agents gradually become less effective, even without washing and, as a result, the cells progress through the cell cycle. During the 26-hr period after the ara-C-treated cells were washed, 35% of the cells proceeded to mitosis, but only about one-half of them yielded colonies. On the other hand, only 4 and 0.2% of the cells treated with 6-MP and 6-MeTPR proceeded to mitosis during the 26-hr period after being washed, but upon extended incubation in normal medium, 35 and 49% (respectively) of the treated cells produced clones. Also, with Meth, 12% more washed cells yielded clones when incubation in normal medium was extended to 7 days. With 5-FdUrd, 5-FUra (3 μg/ml), and 6-TG, only about 9% of the washed cells produced clones during 7 days of incubation in normal medium. No cells survived following a 26-hr period of exposure to 5-FUra at a concentration of 10 μg/ml.

**Evidence of Partial Synchronization.** Chart 4 shows the mitotic indexes of cultures at various times after they had been exposed to the agents for 26 hr and refed with normal medium. The mitotic index of a standard culture is approximately 0.025. With ara-C, HU, and Gz, there were waves of mitoses with indexes that significantly exceeded 0.025. With 5-FUra, the mitotic index rose to supranormal values after 24 hr; and with Meth and 5-FdUrd the mitotic indexes were subnormal throughout the period of observation. 6-MP and 6-MeTPR (data not presented) caused subnormal mitotic indexes throughout the period of observation; the cells that were exposed to 6-TG for 26 hr were in such poor condition that satisfactory preparations of nuclei were not obtained.

The waves of mitoses indicate that partial synchronization of the cultures occurred. The fact that the leading edge of the
Viability and Progression through Cell Cycle

Table 3
Effects of agents upon the progression of cells to mitosis and upon cell survival

<table>
<thead>
<tr>
<th>Agent</th>
<th>During 28-hr exposure</th>
<th>During 26-hr period after 26-hr exposure and washing</th>
<th>% surviving after 26-hr exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C (50 µg/ml)</td>
<td>13</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>HU (50 µg/ml)</td>
<td>15</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td>Gz (500 µg/ml)</td>
<td>16</td>
<td>71</td>
<td>45</td>
</tr>
<tr>
<td>5-FdUrd (10 µg/ml)</td>
<td>13</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Meth (10 µg/ml)</td>
<td>13</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>6-MP (10 µg/ml)</td>
<td>31</td>
<td>4</td>
<td>35</td>
</tr>
<tr>
<td>6-MeTPR (10 µg/ml)</td>
<td>35</td>
<td>0.2</td>
<td>49</td>
</tr>
<tr>
<td>6-TG (10 µg/ml)</td>
<td>13</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>5-FUra (3 µg/ml)</td>
<td>28</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

waves obtained with ara-C and HU were observed at 10 to 11 hr indicates that the blockade of progression of cells occurred near the G₁-S transition point. The concentration of HU in this experiment was 400 µg/ml, whereas in the experiments described above the concentration was 50 µg/ml. In other experiments in which lower concentrations of HU were used, the leading edge of the wave was observed at 6 to 8 hr as was the case with Gz in this and other experiments. It is suspected that the early appearance of this leading edge is due to the disappearance of the HU and Gz from the medium during the 26-hr period of exposure of the cells to the lower concentrations of these agents, which would permit the synchronized cells to proceed through the cycle to some extent before being washed. Although Meth and 5-FdUrd have been used by a number of investigators (48, 55) to achieve synchronization of cultures, waves of mitoses did not occur with these agents in this experiment, because washing does not remove these agents from the enzyme complexes within the cells, and the quantity of thymidine-methyl-³H in the medium was not sufficient to overcome the metabolic blocks.

Chart 5 shows that accumulation of the cells at or near the G₁-S transition occurred with Meth, 5-FdUrd, and 5-FUra (3 µg/ml), because 62, 74, and 80%, respectively, of the cells of these cultures became labeled during the 1st hr of incubation in the presence of thymidine-methyl-³H whereas, in a normal culture, only approximately 20% of the cells became labeled (Table 2, Column 5). Although only approximately 37% of the cells were labeled during the 1st hr after exposure to HU and Gz, by 2 hr, about 85% of the cells were labeled. After the 1st hr, only 7% of the cells that had been exposed to ara-C for 26 hr were labeled but, by 10 hr, 85% of the cells were labeled. In a normal culture about 15 hr would be required to attain this level of labeling. Thus, these 6 agents caused various degrees of synchronization at or near the G₁-S transition.

After treatment of the cells with 6-MP or 6-MeTPR, the labeling curves (not shown) gave no evidence of synchronization, and 14 and 4 hr, respectively, were required for 20% of the cells to become labeled. It is not known whether the cells that did become labeled were in S at the time the thymidine-methyl-³H was added or whether they proceeded from G₁ into S after the labeled substrate was added.
DISCUSSION

When exponential dose-response curves are obtained, the usual interpretation is that the agent kills cells in all phases of the cell cycle (13). When flattened curves are obtained in experiments in which the period of exposure to the agent is short compared with the length of the cell cycle, the usual interpretation is that the agent kills only cells that are in certain phases of the cell cycle and that increasing the concentration of the agent does not increase the cell kill (13).

In the present experiments, the cells were exposed to the agents for a full cell cycle time and, hence, 90% or more of the cells (Table 2) should progress to any sensitive phase of the cycle and be killed, unless the agent also interferes with such progression. The flattened curves obtained here with Meth, ara-C, HU, 5-FdUrd, and 6-MP are consistent with the observations of other investigators (6, 12, 13, 28), who used other cell lines and other procedures. The flattened curves presented here for Gz, 6-TG, and 6-MeTPR are the 1st data reported for these compounds. The different levels at which the curves flatten may reflect the differences in modes of action of the agents, in phase specificity of toxic effects of the agents, in the effects of the agents upon progression through the cycle, and in the ease of removing the agents from the cells by washing (incomplete removal of the agent would lengthen the period of exposure). The exponential curve obtained with 5-FUra, which is in agreement with the observations of others (6, 13, 39), might indicate that this agent kills cells in all phases of the cycle or that it does not interfere with the progression of cells to the sensitive phase of the cycle during the exposure.

There is evidence (8, 16, 18, 45) that agents that specifically inhibit the synthesis of DNA should cause unbalanced growth and, if the state of unbalanced growth is maintained long enough, death of the cells. Of the agents studied in this investigation, ara-C, HU, Gz, and 5-FdUrd specifically inhibit the synthesis of DNA and cause partial synchronization, as shown in this report and in those of others (4, 9, 10, 23, 30, 49, 55). ara-C (10, 15, 57), HU (15, 48), and 5-FdUrd (15, 18, 45) cause unbalanced growth and the formation of giant cells (Gz has not been investigated for formation of giant cells). Under certain conditions, Meth can also specifically inhibit the synthesis of DNA, and it can cause partial synchronization (48, 55) and the formation of giant cells (8, 45). Therefore, one might expect these 5 agents to have similar effects upon the progression of cells through the cycle, and such similarities were indeed observed (Table 2; Chart 5). It has also been shown that cells are most sensitive to ara-C (3, 28), HU (56), and Meth (8) when they are in S phase. Cells in G1, S, and G2 were about equally sensitive to 1 hr of exposure to 5-FdUrd; cells in mitosis were less sensitive (38). Some of the quantitative differences in the effects of these 5 agents are...
perihce due to differences in the stabilities of the agents in the biological media and in the degrees of reversibility of the agent-enzyme interactions.

Although 5-FUra is converted to 5-FdUrd by cells (26) and can cause unbalanced growth and giant cell formation (37), it has toxic effects in addition to those that can be accounted for by this generated 5-FdUrd. Increasing the concentration of 5-FdUrd from 1 to 10 μg/ml did not increase the cell kill of H. Ep. No. 2 (Chart 2), but increasing the concentration of 5-FUra from 1 to 5 μg/ml increased the cell kill from 52 to 99% (Table 2). Although 5-FdUrd at a concentration of 10 μg/ml prevented all cells initially in S from proceeding to mitosis during 1 cell cycle period, 5-FUra at a concentration of 15 μg/ml, which was lethal to all of the cells, allowed cells in the last one-third of S to proceed to mitosis. A 26-hr exposure to 5-FUra at 3 μg/ml, followed by washing, was approximately equivalent to a similar treatment with 5-FdUrd at 10 μg/ml, as determined by the degree of synchronization (Chart 5), progression to mitosis during the subsequent 26-hr period (Table 3), and survival (Table 2). These relative concentrations are significant since, on a molar basis, 5-FdUrd was more than 10,000 times as effective as 5-FUra in inhibiting the incorporation of formate-14C into DNA thymine by Ehrlich ascites cells in vitro (26). The supplementary cytotoxic effects of 5-FUra might be due to its incorporation into RNA or to some as yet undefined role, such as possible interference with the formation and/or functioning of nucleotide oligosaccharides.

If 6-MP, 6-MeTPR, and 6-TG inhibit the de novo synthesis of purine ribonucleotides, one would expect that they would inhibit the synthesis of both DNA and RNA, and such inhibition does occur (20). Therefore, these agents should not cause unbalanced growth, and Pittillo and Rice (44) observed that 6-MP and 6-TG could prevent thymineless death in bacteria. Since the synthesis of RNA occurs throughout the cell cycle except during mitosis (1), it might be expected that these agents would inhibit cells at all parts of the cycle except mitosis. However, they did not prevent cells initially in G2 from progressing to mitosis (Table 2, Column 2), and this is consistent with the failure of actinomycin D to prevent cultured rabbit kidney cortex cells (31) and H. Ep. No. 2 cells (unpublished data from this laboratory) from proceeding to mitosis. Actinomycin D permitted H. Ep. No. 2 cells initially in the last one-half of S to proceed to mitosis (unpublished data), and 6-MP and 6-MeTPR permitted cells initially in all of S to proceed to mitosis, but at slower rates than normal (Table 2). 6-TG permitted cells in G2 (but not those in S) to proceed to mitosis. Like actinomycin D (unpublished data; Ref. 31), these 3 agents did not prevent the labeling of cells initially in S, but they prevented or retarded the migration of cells from G1 to S. These experiments yielded no evidence that these purine analogs caused any accumulation of cells near the G1-S transition point, and it is possible that the progression of the cells was stopped throughout G1. Vandevoorde and Hansen (51) reported that 6-MP arrested cells in G1, and the data of Chart 2 are consistent with the possibilities that 6-MP and 6-MeTPR kill cells initially in G1 and S.

Of the agents included in this study, only 5-FUra would be expected to kill all of the cells by a continuous exposure for 1 cell cycle period. All of the other agents are more toxic to cells in specific phases of the cycle, interfere with the progression of cells through the cycle, and are nonlethal to cells initially in G2. Therefore, in an effort to accomplish total cell kill with any of these agents (except 5-FUra), it would be desirable to have multiple exposures of the cells to the agents with sufficient time and conditions between exposures to allow the cells to progress into the sensitive portions of the cycle. With Meth and 5-FdUrd it might be necessary to use thymidine to overcome the metabolic block of synthesis of thymidylate to allow the cells to progress into S, where they would be sensitive to the subsequent treatment with the agent.

On the other hand, combinations of 2 or more of these agents might be chemotherapeutically beneficial. Several types of combinations that might be worthy of testing are as follows: (a) 2 or more agents that primarily affect cells in the S phase (HU, G2, ara-C, Meth, and 5-FdUrd) by different mechanisms; (b) the agents mentioned in a, plus 6-MP, 6-MeTPR, or 6-TG, which might affect cells primarily in G1; and (c) the agents mentioned in a and/or b, with 5-FUra, which appears to be non-phase specific. Combinations of these agents with non-phase-specific agents (such as ionizing radiation and alkylating agents) might also be effective. Several of these combinations have been (14, 17, 22, 25, 32-34, 46, 47, 52) and are being tested in experimental systems.

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