Enhancement of the Antitumor Activity of 5-Fluorouracil by Drug Combinations

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SUMMARY

We planned the treatment of Ehrlich ascites tumor, taking into account the duration of cell-cycle phases and their sensitivity to various drugs. Two days after the inoculation of $10^7$ ascites tumor cells, mice were given one injection of different drug combinations and the tumor cell number was then counted. It was found that hydroxyurea enhanced the antitumor activity of 5-fluorouracil (5-FU). This effect was evident only when 5-FU was injected together with vinblastine or when 5-FU was injected twice with an interval close to the G2 phase duration. Hydroxyurea alone had no effect on tumor growth. Important features of the enhancement of 5-FU activity by hydroxyurea were the cell death that was evident from the decrease in cell number and significant prolongation of proliferation inhibition. An additive action was demonstrated for 5-FU and vinblastine. Enhancement of the antitumor action by the drug combinations described was not accompanied by increased toxicity.

A combination of drugs that damage different phases of the cell cycle and the injection of drugs with a time interval sufficient for resistant cells to pass into the sensitive phase may prove to be useful.

INTRODUCTION

A rational approach to combination chemotherapy involves the combined use of drugs that damage tumor cells in different phases of the cell cycle. This approach may be especially useful for 5-FU or other antimetabolites to which cells are resistant in certain phases of the cycle. The sensitivity to 5-FU changes during the cell cycle; this follows from 2 investigations on the tissue culture with 5-FUdR. Ockey et al. (11) studied chromosome damage and cell division after treatment of hamster cells with 5-FUdR. Ockey et al. (11) studied chromosome damage and cell division after treatment of hamster cells with 5-FUdR. Lozzio (8) found that the G2 period is insensitive to this drug. Lozzio (8) found that the G2 period is insensitive to this drug. Lozzio (8) found that the G2 period is insensitive to this drug. Lozzio (8) found that the G2 period is insensitive to this drug.

Duration of the cell-cycle phases must also be taken into account in the planning of tumor therapy, especially with antimetabolites (12). As cells in mitosis and the G2 phase may be resistant to 5-FU and cells in the S and G1 phases are sensitive to it, we suggested that it would be efficient to inject a 2nd dose of 5-FU with an interval close to the maximum G2 phase duration. In this way the cells that are not in G0 or S phase at the time of the 1st injection can be affected by the 2nd dose.

It was also interesting to study the combined effect of 5-FU and HU, another inhibitor of DNA synthesis. The mechanisms of action of these inhibitors are different. 5-FU inhibits DNA synthesis by blocking the enzyme thymidylate synthetase, which catalyzes the methylation of dUMP to TMP (5); and HU inhibits reduction of ribonucleoside diphosphate and consequently the formation of dUMP (16).

MATERIALS AND METHODS

The Ehrlich ascites tumor used throughout the study was a hyperdiploid subline grown in noninbred white mice. It was maintained by routine injections of 0.2 ml of ascites fluid at 7-day intervals. In the experiments described below, $10^7$ ascites tumor cells in 0.2 ml of 0.9% NaCl

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1 The abbreviations used are: 5-FU, 5-fluorouracil; 5-FUdR, 5-fluoro-2-deoxyuridine; VB, vinblastine; HU, hydroxyurea.
The dose injected per g of mouse was contained in 0.01 ml. Doses were used: 5-FU, 100 mg/kg VB, 0.25 mg/kg and HU, 300 mg/kg. The effect was significantly higher than with separate injection (Chart 1). When both drugs were injected simultaneously, only 20% higher than for the controls (15.1 ± 0.4).

RESULTS

5-FU, 100 mg/kg, or VB, 0.25 mg/kg, injected on the 2nd day after inoculation retarded the tumor growth (Chart 1). When both drugs were injected simultaneously, the effect was significantly higher than with separate injections of each drug. Injection of the combination of 5-FU, VB, and HU gave the greatest effect (Chart 1). The qualitative difference between the action of this triple combination and that of the other groups was the decrease in the cell number. Injection of HU alone had no effect on tumor growth (Table 3). Since the most marked difference between the various combinations was found on the 5th day after the injection of drugs, a comparison of different drug combinations was made by cell count on this day (Tables 1 to 3).

The effect of the 5-FU and VB combination for both drugs for a significant dose range exceeded that of the high doses of each drug injected separately, for example, of 5-FU in a dose higher than 50% lethality (Tables 1 and 2). As can be seen from Tables 1 and 2, injection of 5-FU, 100 mg/kg, and VB, 0.05 to 1 mg/kg, or of VB, 0.25 mg/kg, and 5-FU, 50 to 100 mg/kg, gave a greater effect than that from 5-FU, 300 mg/kg, or VB, 2 mg/kg, alone. Addition of HU to the combination of 5-FU and VB significantly enhanced the action of this combination for all doses of VB and for 5-FU, 50 to 100 mg/kg. Decrease of the cell number as compared with that observed at the time of treatment occurred only when the 3 drugs were injected simultaneously. HU alone did not change the cell number significantly (Table 3) and did not enhance the effect of VB (Table 2). The effect of the 5-FU and HU combination in the absence of VB was only slightly higher than that of 5-FU alone (Table 1).

An increase in 5-FU activity by HU was found when HU was combined with 5-FU injected in 2 doses with 4- and 7-hr intervals (Table 3). With this combination the enhancement of 5-FU action by HU was observed at a dose of 5-FU, 50 mg/kg, or higher just as for the combination with VB. The inhibition induced by 2 injections of 5-FU plus HU injected simultaneously with the 1st dose greatly exceeded the effect of 5-FU, 300 mg/kg. A decrease in cell number compared with that at the time of treatment occurred when HU was combined with 2 injections of 5-FU, 100 mg/kg (Table 3). It may be seen from Table 3 that the effect of 2 injections of 5-FU was greater for the 7- than for the 4-hr interval.

The enhancing effect of HU was evident with doses of 150 to 300 mg/kg and absent with doses of 37.5 to 75 mg/kg (Table 4). These 2 dose levels differ in their effect on DNA synthesis. Enhancement of the antitumor activity of 5-FU occurs only with doses of HU leading to significant inhibition of DNA synthesis.

As shown in Table 5, several drug combinations displaying significantly enhanced antitumor activity do not show greater toxicity. Single drugs or drug combinations injected twice with a 7-day interval did not kill mice, but with a 5-day interval 2 drug combinations (5-FU + VB and 5-FU + VB + HU) were highly toxic (50% lethality). HU enhanced the toxic effect of a high dose of 5-FU. 5-FU, 250 mg/kg (50% lethality), killed 13 of 25 mice (52%) and 5-FU, 250 mg/kg, plus HU 300 mg/kg, killed 22 of 25 (88%). Injection of HU alone in this dosage was not toxic. Thus the enhancement of the antitumor effect by the above drug combinations was not accompanied by increased
Antitumor Activity of 5-FU and Drug Combinations

Table 1
Effect of combination chemotherapy on the tumor cell number in the ascitic fluid of Ehrlich tumor with dependence on the VB dose

<table>
<thead>
<tr>
<th>VB dose (mg/kg)</th>
<th>Vinblastine</th>
<th>VB + 5-FU, 100 mg/kg</th>
<th>VB + 5-FU, 100 mg/kg + HU, 300 mg/kg</th>
<th>Control (tumor cells x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>202 ± 62</td>
<td>108 ± 27</td>
<td>28 ± 11</td>
<td>1011 ± 175</td>
</tr>
<tr>
<td>1</td>
<td>380 ± 54</td>
<td>87 ± 13</td>
<td>33 ± 8</td>
<td>965 ± 66</td>
</tr>
<tr>
<td>0.25</td>
<td>348 ± 42</td>
<td>134 ± 34</td>
<td>20 ± 7</td>
<td>1039 ± 131</td>
</tr>
<tr>
<td>0.05</td>
<td>326 ± 66</td>
<td>106 ± 13</td>
<td>40 ± 14</td>
<td>660 ± 102</td>
</tr>
<tr>
<td>0.025</td>
<td>540 ± 110</td>
<td>162 ± 16</td>
<td>65 ± 17</td>
<td>660 ± 102</td>
</tr>
<tr>
<td></td>
<td>391 ± 20</td>
<td>75.5</td>
<td>75.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61.1</td>
<td></td>
</tr>
</tbody>
</table>

* Injection of drugs i.p. 2 days after inoculation of 10^6 tumor cells.

Effect of combination chemotherapy on the tumor cell number in the ascitic fluid of the Ehrlich tumor with dependence on the 5-FU dose

Table 2

<table>
<thead>
<tr>
<th>5-FU dose (mg/kg)</th>
<th>5-FU</th>
<th>5-FU + VB, 0.25 mg/kg</th>
<th>5-FU + VB, 0.25 mg/kg + HU, 300 mg/kg</th>
<th>Control (tumor cells x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>244 ± 56</td>
<td>80 ± 30</td>
<td>32 ± 3</td>
<td>96.5</td>
</tr>
<tr>
<td>100</td>
<td>483 ± 28</td>
<td>237 ± 30</td>
<td>80 ± 25</td>
<td>91.1</td>
</tr>
<tr>
<td>50</td>
<td>504 ± 35</td>
<td>196 ± 56</td>
<td>183 ± 10</td>
<td>79.7</td>
</tr>
<tr>
<td>25</td>
<td>380 ± 54</td>
<td>280 ± 78.2</td>
<td>183 ± 10</td>
<td>79.7</td>
</tr>
<tr>
<td>12.5</td>
<td>520 ± 40</td>
<td>307 ± 26</td>
<td>218 ± 24</td>
<td>76.9</td>
</tr>
<tr>
<td>0</td>
<td>900 ± 75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>375 ± 23</td>
<td>400 ± 44</td>
<td>55.6</td>
</tr>
</tbody>
</table>

* Injection of drugs i.p. 2 days after inoculation of 10^6 tumor cells.

DISCUSSION

The 2 main effects described in this paper are the additive action of 5-FU and VB and the enhancement of 5-FU activity by HU. The latter effect was obtained only when 5-FU was combined with VB or when 5-FU was injected twice with an interval close to the maximum G<sub>2</sub> phase duration. The important features of the enhancement of 5-FU activity by HU were the cell death as evidenced from a decrease in cell number and the significant prolongation of proliferation inhibition.

A possible model for explaining these results is proposed.
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Table 4
Effect of different HU doses on the antitumor activity of 5-FU plus VB and on the DNA synthesis

<table>
<thead>
<tr>
<th>HU dose (mg/kg)</th>
<th>Tumor cells × 10^9</th>
<th>% inhibition</th>
<th>Incorporation of thymidine-3H after injection of HU alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% inhibition</td>
</tr>
<tr>
<td>300</td>
<td>20 ± 4</td>
<td>97.1</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td>150</td>
<td>29 ± 7</td>
<td>95.8</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>75</td>
<td>120 ± 37</td>
<td>82.6</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>37.5</td>
<td>115 ± 21</td>
<td>83.5</td>
<td>70 ± 6</td>
</tr>
<tr>
<td>0</td>
<td>124 ± 20</td>
<td>82.2</td>
<td></td>
</tr>
</tbody>
</table>

* Injection of drugs i.p. 2 days after inoculation of 10^7 tumor cells.
* Number of tumor cells 5 days after treatment.
* HU injected 2 days after inoculation of tumor cells; 10 μCi thymidine-3H (4 Ci/m mole) was injected 1 hr later, and mice were killed in 30 min. Autoradiographs were exposed for 7 days.

Table 5
Toxicity of different drug combinations for normal mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wt loss (5th day)</th>
<th>Survived/injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU, 100 mg/kg</td>
<td>–5.7</td>
<td>11/12</td>
</tr>
<tr>
<td>VB, 0.25 mg/kg</td>
<td>–1</td>
<td>12/12</td>
</tr>
<tr>
<td>HU, 300 mg/kg</td>
<td>0</td>
<td>12/12</td>
</tr>
<tr>
<td>5-FU + VB</td>
<td>–5.3</td>
<td>12/12</td>
</tr>
<tr>
<td>5-FU + VB + HU</td>
<td>–6.1</td>
<td>11/12</td>
</tr>
</tbody>
</table>

* Day of maximum weight loss.

5-FU affects cells in the G1 and S phases, but not those in the G2-M phases. The latter continue their movement through the cell cycle and are arrested in mitosis by VB, injected simultaneously with 5-FU. This may explain the additive effect of 5-FU and VB. VB may also affect S-phase cells, escaping the blocking effect of 5-FU; for example, it might affect cells in the later part of the S period with a low sensitivity to fluorinated pyrimidines (11) when they come to mitosis. However, as cells in the S phase may be damaged by VB (10) there might be another mechanism of the additive effect of 5-FU and VB besides that described above.

HU enhanced the antitumor activity of 5-FU only when treatment was scheduled in such a way that the cells expected to be resistant to 5-FU were affected by the 2nd dose of 5-FU or were arrested in mitosis by VB. Evidently, HU enhanced 5-FU action on cells in the S phase. The correlation between the effect of HU on DNA synthesis and the enhancement of 5-FU activity supports this suggestion. Two hypotheses can be made to explain the increase in 5-FU activity by HU. First, the combination of 5-FU and HU can lead to stronger inhibition of DNA synthesis than that induced by each agent alone. Different inhibitors of DNA synthesis lead to cell death only above a certain level of inhibition and a 3% level of DNA synthesis protects the cells from lethality (2). Second, HU may change the metabolism of 5-FU; for example, it might elevate the amount of the lethal nucleotide formed. A study of 5-FU metabolism in the presence of HU might further the understanding of the mechanisms of sensitivity to 5-FU.

5-FU over a significant range of doses gave approximately the same percentage inhibition, but when 5-FU was combined with VB the effect increased with the dose of 5-FU (Table 2). This is consistent with the absence of enhancement of the 5-FU activity by HU when 5-FU is given in a single dose and with the necessity of VB injection for the appearance of such an enhancement. VB even in as small a dose as 0.5 to 1 μg/mouse enhanced the activity of 5-FU and 5-FU + HU (Table 1). This stressed the significance of the combination of drugs affecting the different phases of the cell cycle. This is particularly important if a certain group of cells (e.g., G2-M cells) is completely resistant to the drug. In this situation even a significant increase in the drug dose or enhancement of its action on the sensitive phase alone by another drug will not increase its effect significantly.

The synergistic effect of the 2 DNA synthesis inhibitors 5-FU and HU may be absent with other inhibitors. For example, the antagonistic action on cell multiplication was observed for 2 other DNA synthesis inhibitors (3).

The method used in the present work for estimation of the antitumor effect has some limitations. First, the number of viable cells may be less than the actual cell count. Second, a large increase in inhibition may not significantly change the survival time especially with single treatment if it is followed by rapid regrowth. The beginning of such a regrowth is seen in Chart 1.

In spite of these limitations there is some correlation between the results described above and clinical observations. Indeed, some clinically effective drug combinations contained 5-FU and VB or vincristine (4, 9). Thus, it is possible that the results obtained even with such a simple model may have some real significance.

Successful use of drug combinations is highly dependent on the properties of the cell cycle. There is a significant difference between the cell cycle of the models used, e.g., between a 2-day Ehrlich ascites tumor and the cell cycle in human tumors. The G1 phase of the latter is longer and the percentage of cells in the S phase is smaller (1, 7, 13, 14, 17). Evidently, these features of the cell cycle must be taken into consideration when planning the treatment of human tumors.

REFERENCES

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