ABSTRACT

Since increasing evidence indicates that combination modality of cancer treatment is preferable, and a series of 5-halo-6-phenyl pyrimidinones has been found to induce interferon production and to stimulate a variety of immune responses, several were tested alone or in combination with cyclophosphamide (CY) against B16 melanoma and P388 leukemia. Thus far, 2-amino-5-bromo-6-(3-fluorophenyl)-4(3H)pyrimidinone (ABMFPP) and its sister compound 2-amino-5-bromo-6-(2-fluorophenyl)-4(3H)pyrimidinone (ABOFPP) were found to be superior to other pyrimidinones including 2-amino-5-bromo-6-(6-phenyl)-4-pyrimidinone which is currently under clinical investigation. Neither ABMFPP nor ABOFPP alone had any significant activity against P388 leukemia. However, a marked synergistic effect was observed when a single i.p. injection of CY at 24 hr after tumor inoculation followed by multiple i.p. injections of either ABMFPP or ABOFPP. For instance, the increase of life span was about 180% when animals received both CY (150 mg/kg) and ABMFPP (125 mg/kg/injection) as compared to 100% increased life span when animals received CY alone, and 0% increased life span when animals received ABOFPP alone. Also, 80% of the animals were long-term survivors (>30 days) when animals received the combination therapy as compared to 20% survivors when animals received CY alone. The synergistic effect exhibited by ABMFPP or ABOFPP correlated positively to the initial reduction of tumor burden by CY. The optimal gap between CY and pyrimidine administration was one day. The best therapeutic response was observed when pyrimidinone was given every 4 days for a total of 7 injections; however, other schedules and dosing frequencies also gave significant responses. The synergistic effect was also observed with B16 melanoma when animals received the combination therapy. The significance of these findings, in terms of theoretical consideration as well as drug development, is discussed.

INTRODUCTION

Increasing evidence indicates that combination modality is preferable in the management of experimental and clinical cancers. Immunotherapy has been used in treating several human cancers including lymphoma, leukemia, and solid tumors (10, 14); however, the results were generally marginal and conflicting (10). One of the possible reasons is that an effective immunotherapy requires that the tumor burden of the host be small. Administration of the immunostimulant alone to the host bearing advanced tumor has generally been proven ineffective (1).

Nonspecific active immunotherapy has been found useful in treating cancers (1, 10). Most work has been done with living bacteria and bacterial products in conjunction with surgery and/or chemotherapy (1, 8, 20). Other studies have been extended to synthetic materials such as pyran copolymer (4), tilorone (17), levamisole (18), polyadenyl-polyuridylic (27), etc. A series of pyrimidinones, particularly ABPP (10) have been found to be potent interferon inducers (9, 22, 26). They modulated a variety of immune responses (7, 13, 22, 23), and some of them also had marginal activity against B16 melanoma and spontaneous lung metastases of murine tumors (16). Hence, they warrant further investigation.

MATERIALS AND METHODS

Agents and Vehicles. CY, purchased from Sigma Chemical Co., St. Louis, MO, was dissolved in sterile 0.9% NaCl (saline) solution. Pyrimidinones, including ABPP, ABMFPP, and ABOFPP were made at The Upjohn Company by published procedures (25, 26), and the structures are shown in Chart 1. These compounds were prepared in fine suspension with Sterile Vehicle 100, prepared for laboratory use at The Upjohn Company, Kalamazoo, MI, prior to injection into the animal. Sterile Vehicle 100 is composed of 5 mg of carboxymethylcellulose, 4 mg of polysorbate 80, 9 mg of sodium chloride, and 9 mg of benzyl alcohol in 100-ml volume.

Animals and Tumors. C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice used in the experiments were supplied by Jackson Memorial Laboratory, Bar Harbor, ME, or Charles River Co., Kalamazoo, MI. Inbred male BALB/c × DBA/2 F1 (hereafter called CD2F1) mice were supplied by Charles River Co. or Frederick Cancer Research Center, Frederick, MD. These mice were generally 5 to 6 weeks of age and weighed 18 to 22 g. Groups of 10 mice were housed in metal suspended cages and were given pelleted food and water ad libitum. The B16 melanoma was maintained by continuous s.c. passage in syngeneic C57BL/6 female mice. For the therapeutic experiments, animals received an i.p. injection of 106 cells/mouse. Cells were counted using a hemacytometer. P388 leukemia was maintained by continuous i.p. passage in syngeneic female DBA/2 mice. This tumor was inoculated i.p. in CD2F1 mice (106 cells/mouse) for the experiments.

Experimental Design. Tumor cells, either B16 melanoma or P388 leukemia, were inoculated i.p. on Day 0. One day later, the animal received a single i.p. injection of CY. The pyrimidinone was administered i.p. starting on Day 2, 4, or 7 and was given weekly thereafter to the B16-bearing animals for a total of 7 injections. The experiment was terminated on Day 60. With P388, the animals also received the injections starting on Days 2, 4, or 7 and either every 2 days, every 4 days, or weekly thereafter for a maximum of 7 injections. The experiment was terminated on Day 30.

The therapeutic response was measured as median survival time (days) of 10 mice/group according to the procedure of Schabel et al. (21) in which the median life span was determined with the dying animals only. The long-term survivors (cures?), in this case 30-day survivors for

1 A portion of this investigation was reported at the 74th Annual Meeting of the American Association for Cancer Research (12).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: ABPP, 2-amino-5-bromo-6-phenyl-4-pyrimidinone; CY, cyclophosphamide; ABMFPP, 2-amino-5-bromo-6-(3-fluorophenyl)-4(3H)pyrimidinone; ABOFPP, 2-amino-5-bromo-6-(2-fluorophenyl)-4(3H)pyrimidinone; IL5, increase of life span.
P388 leukemia and 60-day survivors for B16 melanoma, were recorded separately. Percentage of ILS was calculated as:

\[
\frac{(\text{Median survival time (days) of treated group}) - 1}{(\text{Median survival time (days) of control group})} \times 100
\]

According to the National Cancer Institute, the criterion for significant therapeutic effect is ≥25% ILS for B16 melanoma (i.p.) and ≥20% for P388 leukemia (i.p. or i.v.).

Acute drug toxicity was estimated by the early deaths of animals and the change in animal body weight which was measured on Days 0, 5, and 11 for BD2F, mice bearing B16 melanoma and on Days 1, 5, and 9 for CD2F, mice bearing P388 leukemia.

**Statistical Analysis.** Median and mean life spans were calculated after removing survivors (>30 and/or >40 days for P388 leukemic mice and >11 days for P388 leukemic mice) and spuriously low life spans. With the log of the life span, spuriously low life spans for the control group were removed using Dixon’s gap test (3). Any life span of a treated group which was <3 S.D. below the control mean was also excluded.

Drewinko et al. (6) called the combined effect of 2 treatments on human lymphoma cells additive if the proportion (treatment divided by control) of surviving cells under combined therapy equaled the product of the proportions under the respective single therapies. If the first proportion was less than the latter product of proportions, the combined effect was called synergistic. Schabel et al. (21) showed that the median life span with survivors excluded was linearly related to the log of the percentage of cell kill. Consequently, the definition of additivity of Drewinko et al. (6) is equivalent to the sum of the median life spans of the control and combined therapies equaling the sum of the median life spans of the 2 single therapies. Since the distribution of life spans is roughly symmetrical after survivors are excluded, the medians can be replaced by the means in this definition. The F test (11) for the equality of these 2 sums, expressed as means, was used to test for synergy.

**RESULTS**

**Combination Therapy of B16 Melanoma.** When animals received an inoculation of approximately 10^6 cells, the median survival time was between 23.5 and 25.5 days. The levels of CY, by design, yielded a modest but significant antitumor effect, e.g., 30 to 40% ILS. Some pyrimidinones alone, such as ABOFPP, produced a small but significant activity (i.e., ≥25% ILS) against B16 melanoma (Table 1). The results indicate that, although the therapeutic effect of CY and ABPP combination treatment appeared to be better than either agent alone, the effects were generally no more than additive (Table 1). With ABOFPP, however, the situation was somewhat different. The combined effect of CY (200 mg/kg) followed by 7 weekly injections of ABOFPP (500 mg/kg/injection) produced a 108% ILS which appeared to be more than the addition of the effects of either CY (35% ILS) or ABOFPP (26% ILS) alone. The acute toxicity, measured by body weight change, appeared to be enhanced with the combination treatment, but no early animal deaths were recorded. As a matter of fact, 4 of the 10 animals survived up to the termination of the 60-day experiment (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Dose (mg/kg/injection)</th>
<th>Body wt changeb (g)</th>
<th>ILS (%)</th>
<th>60-day survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Vehicle 100</td>
<td>0 0</td>
<td>0.8 0</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>CY + Sterile Vehicle 100</td>
<td>200 0</td>
<td>-2 -2</td>
<td>35 35</td>
<td>1/10</td>
</tr>
<tr>
<td>ABPP</td>
<td>0 50</td>
<td>1.0 10</td>
<td>18 18</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>200 50</td>
<td>-1.7 49</td>
<td>49 49</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>0 125</td>
<td>1.2 16</td>
<td>16 16</td>
<td>0/10</td>
</tr>
<tr>
<td>ABOFPP</td>
<td>200 125</td>
<td>-3.2 41</td>
<td>41 41</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>0 125</td>
<td>1.4 26</td>
<td>26 26</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>200 125</td>
<td>-1.8 78</td>
<td>78 78</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>0 250</td>
<td>1.0 26</td>
<td>26 26</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>200 250</td>
<td>-3.1 69</td>
<td>69 69</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>0 500</td>
<td>0.9 26</td>
<td>26 26</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>200 500</td>
<td>-3.8 108</td>
<td>108 108</td>
<td>4/10</td>
</tr>
</tbody>
</table>

a Tumor (approximately 10^6 cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. Sterile Vehicle 100 or pyrimidinone was injected i.p. on Day 2 and weekly thereafter for a total of 7 injections.

b Change per animal, measured on Day 5.

c Median death of untreated tumor-bearing animals (control) was 25.5 days.

**Table 2**

<table>
<thead>
<tr>
<th>Combination therapy of P388 leukemia with CY and pyrimidinones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatmenta</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Sterile Vehicle 100</td>
</tr>
<tr>
<td>CY + Sterile Vehicle 100</td>
</tr>
<tr>
<td>ABPP</td>
</tr>
<tr>
<td>CY + ABPP</td>
</tr>
<tr>
<td>ABBFPP</td>
</tr>
<tr>
<td>CY + ABBFPP</td>
</tr>
<tr>
<td>ABBFPP</td>
</tr>
<tr>
<td>CY + ABBFPP</td>
</tr>
<tr>
<td>ABOFPP</td>
</tr>
<tr>
<td>CY + ABOFPP</td>
</tr>
</tbody>
</table>

a Tumor (10^6 cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. Sterile Vehicle 100 or pyrimidinone was injected i.p. on Day 2 and every 4 days thereafter for a total of 7 injections.

b Change per animal, measured on Day 5.

c Median death of vehicle-treated tumor-bearing animals was 11 days (range, 10 to 12 days).

d p < 0.001 by F test for synergy based on the comparison of mean life span of 40-day nonsurvivors (range, 24 to 39 days).
Combination Therapy of P388 Leukemia. When CDF₁ mice received a single i.p. injection of CY (150 mg/kg) 24 hr after tumor inoculation (10⁶ cells/mouse) followed by 7 i.p. injections of the pyrimidinone at a 4-day interval, the combination effect of CY and ABPP again was no more than the effect of CY used alone (Table 2). However, a synergistic effect was clearly observed when CY was used in conjunction with either ABMFPP or ABOFPP. An approximately 180% ILS was observed when tumor-bearing animals received both CY (150 mg/kg) and ABMFPP (125 mg/kg/injection) or ABOFPP (500 mg/kg/injection) as compared to 109% ILS when animals received CY alone and 0% ILS when animals received either pyrimidinone alone (Table 2). The synergistic effect was highly significant statistically (p < 0.001). Furthermore, at least 90% of the animals receiving the combination therapy were long-term survivors (>30 days) as compared to 20 and 0% of long-term survivors in animals receiving either CY or pyrimidinone alone, respectively. The synergistic effect was also observed with the use of a lower dose of CY (75 mg/kg). Animals that received both CY and ABMFPP (125 mg/kg/injection) or ABOFPP (500 mg/kg/injection) gave ILSs of 100 and 88% as compared to an ILS of 58% when the animals received CY alone (Table 3). Again, the differences were highly significant statistically; however, no significant number of long-term survivals was noted. A significant synergistic effect was also observed with the combination of CY and ABPP (100 mg/kg/injection); however, the effect was not as great as was the effect with ABMFPP or ABOFPP. The magnitude of synergism appeared to be related to the dosage of the pyrimidinone. For instance, at a given dose of CY (100 mg/kg), no significant synergism was observed when the dose of ABMFPP was lower than 62.5 mg/kg/injection. At 250 mg/kg/injection of ABMFPP or higher, the combination treatment appeared to be somewhat toxic to the animal as judged by body weight loss (data not presented). Hence, the optimal dose of ABOFPP (500 mg/kg/injection) or ABMFPP (125 mg/kg/injection) was used throughout the study.

Table 3
Combination therapy of P388 leukemia with CY and pyrimidinones

<table>
<thead>
<tr>
<th>Dose (mg/kg/injection)</th>
<th>Treatment</th>
<th>CY</th>
<th>Pyrimidinone</th>
<th>ILS (%)</th>
<th>30-day survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Vehicle 100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td>CY + Sterile Vehicle 100</td>
<td>75</td>
<td>50</td>
<td>58</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td>ABPP</td>
<td>75</td>
<td>50</td>
<td>63</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>100</td>
<td>75</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td>ABMFPP</td>
<td>75</td>
<td>125</td>
<td>100</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td>ABOFPP</td>
<td>75</td>
<td>250</td>
<td>0</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>250</td>
<td>75</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>500</td>
<td>0</td>
<td>0%</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>500</td>
<td>86</td>
<td>0%</td>
<td>1/10</td>
</tr>
</tbody>
</table>

* Tumor (10⁶ cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. Sterile Vehicle 100 or pyrimidinone was injected i.p. on Day 1 and every 4 days thereafter for a total of 7 injections.

Determination of the Optimal Gap between CY and Pyrimidinone Administration. Since the gap between the administration of the cytotoxic agent and immunostimulants may have a marked influence on the outcome of the adjuvant therapy, we decided to investigate this aspect promptly. With B16 melanoma, CY was injected on Day 1, while the first injection of ABOFPP was administered on Day 2, 4, or 7 and weekly thereafter for a total of 7 injections for all the treatments. The combination effect was highest (96% ILS) when the first pyrimidinone injection was given on Day 2. The synergistic effect was reduced when ABOFPP was given on Day 4 and was almost eliminated when ABOFPP was given on Day 7 (Table 4). ABOFPP alone also produced a small but significant anti-B16 melanoma activity (≥25% ILS).

Similar results were obtained with P388 leukemia when the animals received CY on Day 1 and ABMFPP on Day 1, 2, 4, or 8. ABMFPP was given every 4 days thereafter for a total of 7 injections. The best therapeutic effect was obtained when the animals received ABMFPP starting on Day 2. The synergistic effect was sharply reduced when ABMFPP was given on Day 8. Overall, the data indicate that the optimal gap between CY and

Table 4
Determination of the optimal gap between CY and pyrimidinone administration

<table>
<thead>
<tr>
<th>Tumor (10⁶ cells/mouse)</th>
<th>Dose (mg/kg/injection)</th>
<th>ILS (%) on SV100²</th>
<th>pyrimidinone injection schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16 melanoma</td>
<td>CY + SV100</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>CY + ABOFPP</td>
<td>200</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>CY + ABOFPP</td>
<td>200</td>
<td>96</td>
</tr>
<tr>
<td>P388 leukemia</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>123</td>
<td>0</td>
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<td></td>
<td>0</td>
<td>118</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Median death of vehicle-treated tumor-bearing animals (controls) are 27.5 days for B16 melanoma and 10.5 days for P388 leukemia.

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the pyrimidinone is one day in treating both experimental tumors.  

**Effect of Dosing Interval and Dosing Frequency of Pyrimidinone Administration.** When the pyrimidinone was given every 2 days, the combination effect with CY was significantly less than when the pyrimidinone was given every 4 days (Table 5). For instance, a 177% ILS was obtained when animals received CY (150 mg/kg) and a total of 7 injections of ABMFPP (125 mg/kg/injection) given every 4 days as compared to only 136% ILS when animals received ABMFPP every 2 days. In another experiment (terminated on Day 30), no significant difference was noted in terms of ILS between those animals receiving 5 to 7 injections of ABMFPP or ABOFPP every 4 days and those receiving 3 to 4 weekly injections of the pyrimidinone. For example, approximately 150% ILS was obtained when animals received CY and 5 to 7 injections of ABMFPP every 4 days or 3 injections of ABMFPP weekly (Table 6); however, the long-term survivors were more noticeable with the every-4-days injection schedule. The combination effect was diminished when animals received less than a total of 5 injections of ABOFPP when given every 4 days (data not presented).

**Effect of Tumor Burden on the Adjuvant Therapy with Pyrimidinones.** The therapeutic effect exhibited by either ABMFPP or ABOFPP was enhanced by the magnitude of initial tumor burden reduction by CY which was indirectly measured by the percentage of ILS as shown in Chart 2. For instance, ABOFPP, at 500 mg/kg/injection for a total of 7 injections, had no apparent therapeutic effect against P388 leukemia with a 0% ILS. A dose of CY at 25 mg/kg produced a 40% ILS. The combination treatment with CY and ABOFPP, however, gave a 50% ILS. The difference between the combination therapy and CY alone was statistically significant (p < 0.05). When a dose of CY (75, 100, or 150 mg/kg) was injected into the tumor-bearing animals, it yielded a 58, 82, and 109% ILS, respectively. The addition of ABOFPP at 500 mg/kg/injection to CY administration at the doses aforementioned yielded a corresponding 88, 136, and 182% ILS. Hence, the ILS value was enhanced by approximately 30, 54, and 73% due to the addition of ABOFPP to the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CY</th>
<th>Pyrimidinone</th>
<th>ILS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CY + SV100</td>
<td>100</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>ABMFPP</td>
<td>0</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>CY + ABMFPP</td>
<td>100</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>ABOFPP</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>CY + ABOFPP</td>
<td>100</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

- a Tumor (10⁶ P388 leukemic cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. The pyrimidinone was injected i.p. starting on Day 2 and was given either every 2 days or 4 days thereafter for a total of 7 injections.
- b Median death of vehicle-treated mice was 11 days.
- c p < 0.001 by F test for synergy based on the comparison of mean life span of 30-day nonsurvivors.

**Table 6** Effect of dosing interval and dosing frequency of pyrimidinone administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/injection)</th>
<th>ILS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CY + SV100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>ABMFPP</td>
<td>0</td>
<td>125</td>
</tr>
<tr>
<td>CY + ABMFPP</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>ABOFPP</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>CY + ABOFPP</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

- a Tumor (10⁶ P388 leukemic cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. The pyrimidinone was injected i.p. on Day 2 and 4 days thereafter for a total of injections as indicated.
- b SV100, Sterile Vehicle 100.
- c x, number of injections.
- d Median death of vehicle-treated tumor-bearing animals was 11 days.
- e p < 0.001 by F test for synergy based on the comparison of mean life span of 30-day nonsurvivors.
- f p < 0.01 by F test for synergy based on the comparison of mean life span of 30-day nonsurvivors.
corresponding dose level of CY. The synergistic effects of CY and ABOFPP were highly statistically significant (p < 0.001). Similar results were obtained with the CY and ABMFPP combination (Chart 2).

The reduction of tumor burden can also be estimated by measuring the killing of P388 leukemia cells through a titration curve which was constructed based on the direct relationship between the number of tumor cells inoculated and the life span of these animals (Chart 3); our results, on the whole, reproduce those of Schabel et al. (21). Since the error of correlation between tumor inoculant and life span of the host increases sharply as the tumor inoculant becomes small, the reduction of tumor cell population, as presented in Table 7, was estimated through the titration curve constructed after several hundred testings by Schabel et al. (21), rather than ours. The results shown in Table 7 indicate that when animals received a dose of CY at 25 mg/kg, the tumor inoculate was reduced from 10^6 cells/mouse to approximately 2.8 x 10^3 cells which needed 17 days to reach the critical tumor cell population (~10^9 cells/mouse) to kill the host animal. The addition of ABMFPP (500 mg/kg/injection) further reduced the tumor load to approximately 10^2 cells/mouse, although no long-term (>30-day) survivors were observed. The addition of ABMFPP (125 mg/kg/injection) reduced the tumor population to an average of less than one cell/mouse and increased the median survival time by 23 days, and 60% of the animals survived longer than 30 days. A dose of CY at 150 mg/kg produced a 5-log reduction of tumor population, and 20% of the animals were long-term survivors; however, the addition of either ABMFPP or ABOFPP further reduced the tumor load to an average of less than 1 cell/mouse, and over 80% of the animals were long-term survivors (Table 7).

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Treatment</th>
<th>Drug dose (mg/kg/injection)</th>
<th>Expected median death (day)</th>
<th>Estimated surviving cells^a</th>
<th>30-day animal survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Vehicle</td>
<td>CY</td>
<td>0</td>
<td>10^11</td>
<td>~10^3</td>
<td>0/10</td>
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<tr>
<td>ABMFPP</td>
<td>100</td>
<td>125</td>
<td>10^11</td>
<td>~10^6</td>
<td>0/10</td>
</tr>
<tr>
<td>CY</td>
<td>150</td>
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<td>10^11</td>
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<td>~10^10</td>
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^a Tumor (10^6 cells/mouse) was inoculated i.p. on Day 0, and CY was injected i.p. on Day 1. The pyrimidinone was injected i.p. on Day 2 and every 4 days thereafter for a total of 7 injections.

^b Calculated from a titration curve constructed by Schabel et al. (21).

DISCUSSION

Immunotherapy, particularly nonspecific active immunotherapy, has been demonstrated to be useful in the treatment of experimental and clinical cancers (1, 10, 14). Although a variety of immunostimulants (immunomodulators or biological response modifiers) have been identified and studied (1, 14), agents having a defined chemical structure that can be reproduced in quality and quantity are more desirable. A series of pyrimidinones as described in the "Introduction" appear to meet the criterion. ABMFPP and ABOFPP were chosen from among 12 compounds tested (data not presented) because of their initially favorable antitumor responses. ABPP, a well-studied pyrimidinone, was included for comparison purposes.

The rationale for the sequential combination of chemoimmunotherapy is illustrated in Chart 4. When a clinical cancer is detected, generally the disease is already in the advanced stages, e.g., >10^9 cells (5). The tumor mass should initially be reduced as much as possible by using surgery, radiation, and/or chemotherapy, since immunotherapy appears to be most
effective when the tumor burden is small (1). CY was used in this study because it has been well-studied and is effective in treating a variety of experimental and clinical cancers. In order to minimize the complexity of the experimental design, a single i.p. injection of CY was given at a dose that produced a desirable therapeutic effect. Pyrimidine was given sequentially, since the concomitant administration was not superior to the sequential injection (Table 4). Pyrimidinone appeared to be more effective when given one day after CY administration. It is reasonable to speculate that the close gap between CY and pyrimidinone administration reduces the opportunity for the residual tumor cell population to rebound after CY treatment. The close gap may also reflect the growth characteristics of P388 leukemia cells, and the optimal gap may vary with different tumors.

Since our primary concern was the effect of tumor burden on the outcome of the sequential combination therapy, various dosages of CY were used in order to reduce the tumor burden initially to the desirable level. A synergism was observed at all levels of CY used followed by pyrimidinone administration at optimal doses (e.g., 125 mg/kg/injection for ABMFPP or 500 mg/kg/injection to ABOFPP) (Chart 2). The effects of pyrimidinones were proportionally enhanced as the initial tumor burden was reduced by CY, which was expressed as a percentage of ILS (Chart 2).

The aforementioned results clearly demonstrate that the theoretical consideration for this therapeutic approach is reasonable. Immunotherapy can make significant contributions to the management of cancers, providing the tumor burden is sufficiently small and the host is able to respond to the immunostimulant and also to tumor-associated antigens (1). In P388 leukemia, the tumor burden must be reduced to less than 10^3 cells/mouse before pyrimidinone can exhibit a significant therapeutic effect (Table 7). Very likely, the obligatory reduction of the initial tumor burden through surgery, radiation, and/or chemotherapy needed for a subsequently successful immunotherapy may vary with different tumor systems and/or different immunostimulants and may also depend on the immune competency of the host. In the clinical situation, the term "remission" implies nothing more than the lower level of detectability of a tumor mass that occurs when it reaches about 1 cu cm in size or 1 g in weight containing approximately 10^8 or one billion cells (5). Thus, even small cancers at the limit of detection or remission may represent far-advanced diseases in a true sense. Based on the information generated here, it is not surprising that so many immunotherapy trials have failed. Until more sensitive and accurate methods are available for determining tumor burden or tumor reduction clinically, it will always be a major obstacle for successful cancer therapy in general and immunotherapy in particular.

With regard to this investigation, several questions remain to be answered. (a) In addition to its immunosuppressive effect, CY has been reported to augment immunity by depleting suppressor T-cells and suppressor monocytes (2, 24). Whether this desirable feature is one of the main reasons for the successful combination therapy remains obscure. The combination of pyrimidinone with other clinically useful cytotoxic antitumor agents which may shed some light on this subject is currently under investigation. (b) The mechanism of action of pyrimidinones and whether the induction of interferon (9, 22, 23) and/or immunostimulation of a variety of immune responses (7, 13, 22, 23) are related to its antitumor effect are also under investigation. (c) Whether pyrimidinones exhibit any effect on suppressor T-cells should also be studied, since bacillus Calmette-Guérin has been reported to induce suppressor cell amplification in addition to the modulation of other immune responses (15).

Clearly, pyrimidinones have been shown to be effective anti-tumor agents when used appropriately in conjunction with the chemotherapeutic modality. ABMFPP was more potent than ABOFPP in the treatment of P388 leukemia (Table 2); however, ABOFPP seemed somewhat more effective than ABMFPP in treating B16 melanoma (data not presented). Whether the differential effects of ABMFPP and ABOFPP on different tumor systems are significant will be evaluated with other experimental tumors. Although these pyrimidinones are most effective when the residual tumor burden is small, it is this small residual tumor population that is enormously difficult to eradicate via the conventional therapy. Ideally, chemotherapy should be continued until the last tumor cell is killed. In most cases, this probably will not occur without involving the patient's own defense system, as pointedly discussed by Pratt and Rudder (19). Our results clearly indicated that immunostimulants, such as pyrimidinones, if used properly, can fulfill the mission without causing apparent severe toxicity to the host and, hence, deserve further development as an adjuvant therapy to surgery, radiation, and/or chemotherapy.

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