The Effect of *Corynebacterium parvum* in Combination with 5-Fluorouracil, L-Phenylalanine Mustard, or Methotrexate on the Inhibition of Tumor Growth

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**SUMMARY**

Previous reports from this laboratory have demonstrated conclusively that cyclophosphamide administered asynchronously with *Corynebacterium parvum* (CP) results in greater C3H mammary tumor inhibition than that observed with either agent alone. An analysis of this combination has revealed that the chemotherapeutic component contributes more significantly to tumor inhibition than does the immunotherapeutic one.

This study was conducted to investigate the inhibition of C3H mammary tumors by other chemotherapeutic agents when used with CP. The results have demonstrated that 60 mg of cyclophosphamide per kg, 90 mg of 5-fluorouracil per kg, and 10 mg of L-phenylalanine mustard per kg administered weekly have similar tumor-inhibiting properties. The addition of CP enhanced the tumor-inhibiting properties of each agent but to differing degrees. The effect of the immunopotentiator when used in combination with alkylating agents was greater than that seen when it was used with the antimetabolite 5-fluorouracil. The tumor inhibition observed when cyclophosphamide was administered asynchronously with CP was significantly greater than that observed when L-phenylalanine mustard was similarly used. Of particular interest was the finding that the addition of CP to a combination of chemotherapeutic agents resulted in no greater tumor growth inhibition than that which occurred when CP was used along with the most effective single agent in the combination. The data have indicated that, contrary to clinical impression, there is no evidence that CP through its toxicity-sparing effect permits the utilization of larger doses of chemotherapy. Consideration has been given to the mechanisms that might account for the differences in tumor growth inhibition encountered when CP was used with different chemotherapeutic agents.

**INTRODUCTION**

Prolonged administration of CP and CY inhibits the growth of established, measurable C3H tumors to a greater extent than does either agent alone (9, 12). In a series of investigations evaluating this model system, information was obtained to indicate that tumor growth deterrence is a result of both the chemotherapeutic components (11). It is pertinent to determine the effectiveness of other chemotherapeutic agents used with CP. Such information could be of value in planning chemotherapeutic and immunotherapeutic treatment regimens for use against human tumors. This report presents results obtained from investigations utilizing 5-FU, L-PAM, or MTX with CP.

**MATERIALS AND METHODS**

**Mice.** Mice used in this study were inbred C3HeB/FeJ females, 8 to 12 weeks of age.

**Tumor.** The tumor used was a spontaneous mammary carcinoma arising in a C3H/HeJ female and was maintained by transfer in C3HeB mice. Tumor cell suspensions were prepared by mincing tumor fragments with scissors on an 80 mesh nylon screen and by washing the cells through the screen with Medium 199. The cells were counted, using trypan blue exclusion as a test of viability. The suspension was then diluted in Medium 199 until it contained 200,000 viable cells in 0.1 ml. In all experiments, tumors were transferred by inoculating 2 × 10⁵ viable tumor cells s.c. into the left hind leg distal to the popliteal node. A tumor approximately 5 mm in diameter developed within 14 days. The longest diameter of the tumor was measured using a Vernier caliper, and the same diameter was used for all subsequent measurements during the course of an experiment. All observations in an experiment were made by the same person. Prior to the beginning of treatment, all tumors were measured. Mice were assigned to various groups in the experiment so that each contained tumors of equivalent size. All mice were weighed and the tumor diameter and health of the animal was noted just prior to each injection. Control mice received 0.9% NaCl solution by the appropriate route at the time when treatments were given to test animals.

**CP.** Burroughs Wellcome CP CN6134³ was used in a dose of 1.4 mg, dry weight of organisms, i.p. every 7 days and 4 days after each dose of chemotherapy. The route of administration, the dose used, and the timing of CP administration have been found in prior investigations by us (9, 11, 12) to be optimal. No experiment includes animals treated with CP alone since extensive evaluation docu-

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¹ Supported by USPHS Grants CA 14972, CA 12102, and CA 13289.
² The abbreviations used are: CP, *Corynebacterium parvum*; CY, cyclophosphamide; 5-FU, 5-fluorouracil; L-PAM, L-phenylalanine mustard; MTX, methotrexate.
³ Supplied by Dr. J. D. Whisnant of Burroughs Wellcome & Co., Research Triangle Park, N. C. 27709.
Chemotherapeutic Agents. Drugs were prepared immediately before use and were diluted in 0.9% NaCl solution (CY in distilled water) so that the desired amount was contained in 0.01 ml/g body weight. They were administered every 7 days. Sixty mg of CY per kg body weight, 60 or 90 mg of 5-FU per kg body weight, and 10 or 15 mg of L-PAM (Alkeran; Burroughs Wellcome) per kg body weight were injected i.p. The latter was refrigerated during use. Fifty or 12.5 mg of MTX per kg body weight were inoculated s.c. at sites on the abdomen or back.

In experiments combining CY with 5-FU, MTX, or L-PAM, the CY was given between 9 and 10 a.m. and the other agent was given between 4 and 5 p.m. When 5-FU was combined with L-PAM, the 5-FU was given in the morning and the L-PAM was given in the afternoon. When MTX was added to the CY-5-FU combination, it was injected 3 or 4 hr after the CY and 3 to 4 hr prior to the 5-FU.

Data relating to tumor size were analyzed using the Student t test.

RESULTS

Effect of CP and 5-FU on Tumor Growth. When 60 mg of 5-FU per kg were administered weekly to tumor-bearing mice, no significant inhibition of tumor growth was observed. A slight but statistically significant effect was obtained when 90 or 120 mg/kg were used (Table 1). The use of 120 mg/kg did, however, result in the death of more than one-half of the mice. Following addition of CP to the treatment regimen, utilizing any dose level of 5-FU, little further decrease in tumor growth occurred. However, the slightly greater effect following the addition of CP to 90 mg of 5-FU per kg was consistently statistically significant. The addition of CP failed to protect mice against the lethal effects of 5-FU. In fact, its use resulted in increased mortality. Deaths in animals receiving 5-FU with or without CP were a result of drug toxicity rather than of tumor growth.

Tumor growth inhibition resulting from 90 mg of 5-FU per kg administered weekly was equivalent to that resulting from the use of 60 mg of CY per kg (Table 2). The addition of CP to CY resulted in a significantly greater inhibition of tumor growth than that which occurred when CP was used with 5-FU (Table 2). In addition, whereas no tumor arrest or regressions were observed when the latter combination was used, with CY and CP, 35% showed a decrease in tumor size and an additional 31% had an arrest of tumor growth for 1 to 3 weeks.

The use of 5-FU in combination with CY resulted in inhibition of tumor growth that was significantly greater than that occurring when CY was used alone (Chart 1). Addition of CP to the combination of 5-FU and CY failed to enhance tumor growth inhibition. The combination of CY and CP, however, was not significantly more effective than CY alone.

Table 1

<table>
<thead>
<tr>
<th>Wk of treatment</th>
<th>Group I (0.9% NaCl solution)</th>
<th>Group II (5-FU)</th>
<th>Group III (5-FU + CP)</th>
<th>Group I (0.9% NaCl solution)</th>
<th>Group II (5-FU)</th>
<th>Group III (5-FU + CP)</th>
<th>Group I (0.9% NaCl solution)</th>
<th>Group II (5-FU)</th>
<th>Group III (5-FU + CP)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3 ± 1.46a</td>
<td>5.0 ± 1.51</td>
<td>5.2 ± 1.60</td>
<td>5.3 ± 1.45</td>
<td>5.3 ± 1.37</td>
<td>5.3 ± 1.42</td>
<td>6.0 ± 1.37</td>
<td>6.0 ± 1.37</td>
<td>6.1 ± 1.05</td>
</tr>
<tr>
<td>4</td>
<td>23.5 ± 4.38</td>
<td>21.9 ± 4.97</td>
<td>17.3 ± 4.97</td>
<td>23.4 ± 4.64</td>
<td>17.7 ± 4.02</td>
<td>15.4 ± 4.04</td>
<td>22.9 ± 4.04</td>
<td>17.0 ± 4.04</td>
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<tr>
<td></td>
<td>(2)</td>
<td>(1)</td>
<td>(4)</td>
<td>(17)</td>
<td>(3)</td>
<td>(6)</td>
<td>(7)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(11)</td>
<td>(6)</td>
<td>(6)</td>
<td>(45)</td>
<td>(3)</td>
<td>(10)</td>
<td>(7)</td>
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<td></td>
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</table>

a Mean ± S.D.

b Numbers in parentheses, number of dead mice.

c Group II vs. Group III. p < 0.01.

Table 2

<table>
<thead>
<tr>
<th>Wk of treatment</th>
<th>Group I (0.9% NaCl solution)</th>
<th>Group II (CY)</th>
<th>Group III (CY + CP)</th>
<th>Group IV (5-FU)</th>
<th>Group V (5-FU + CP)</th>
<th>Group II vs. Group III</th>
<th>Group IV vs. Group V</th>
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<tr>
<td>0</td>
<td>5.3 ± 1.24a</td>
<td>5.5 ± 1.45</td>
<td>5.5 ± 1.23</td>
<td>5.6 ± 1.28</td>
<td>5.6 ± 1.34</td>
<td>0.8</td>
<td>0.7</td>
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<td>4</td>
<td>24.5 ± 3.84</td>
<td>16.0 ± 4.02</td>
<td>10.1 ± 3.61</td>
<td>17.2 ± 3.53</td>
<td>15.5 ± 4.57</td>
<td>0.001</td>
<td>0.2</td>
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<tr>
<td>6</td>
<td>26.1 ± 1.91</td>
<td>20.0 ± 3.26</td>
<td>12.5 ± 4.36</td>
<td>22.9 ± 4.22</td>
<td>20.4 ± 4.55</td>
<td>0.001</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>(38)</td>
<td>(6)</td>
<td>(7)</td>
<td>(3)</td>
<td>(6)</td>
<td>(9)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

a 48 mice/group. 5-FU (90 mg/kg), CY (60 mg/kg), and CP (1.4 mg).

b Mean ± S.D.

c Numbers in parentheses, number of dead mice.
Effect of addition of CP to L-PAM on tumor growth and survival

Table 3

<table>
<thead>
<tr>
<th>Wk of treatment</th>
<th>Group I (0.9% NaCl solution)</th>
<th>Group II (L-PAM)</th>
<th>Group III (L-PAM + CP, 1.4 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.9 ± 0.98^a</td>
<td>5.0 ± 0.87</td>
<td>5.1 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>20.4 ± 3.28 (4)^a</td>
<td>16.5 ± 3.03 (1)</td>
<td>12.2 ± 3.32 (10)</td>
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<tr>
<td></td>
<td>25.9 ± 3.12 (13)</td>
<td>19.5 ± 3.88 (3)</td>
<td>14.6 ± 3.93 (19)</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.
^b Numbers in parentheses, number of dead mice.
^c Group II vs. Group III, P < 0.01.

An experiment was conducted to compare directly tumor growth inhibition resulting from L-PAM and CP with that occurring following the use of CY and CP (Chart 3). The degree of tumor growth inhibition following 10 mg of L-PAM per kg was not significantly different from that resulting from the use of 60 mg of CY per kg. The addition of CP to L-PAM significantly increased the effect of L-PAM in that regard, but to a lesser degree than when CP was combined with CY.

A comparison of tumor growth occurring following CP and L-PAM treatment was made with that which resulted after CP and 5-FU administration (Table 4). A greater increment of tumor growth inhibition resulted from the addition of CP to L-PAM.

Effect of CP and MTX on Tumor Growth. When 50 mg of MTX per kg (the maximum dose tolerated) were administered weekly to tumor-bearing mice, some inhibition of growth inhibition beyond that resulting from the use of CP and CY alone (Chart 1). There was no statistically significant difference in tumor growth between the groups that received CY + 5-FU, CY + 5-FU + CP, and CY + CP. An additional investigation revealed that the addition of CP to a 3-drug combination (CY + 5-FU + MTX) resulted in tumor growth inhibition that was no greater than that which occurred following the use of CY and CP (Chart 2). Moreover, the addition of CP to the combination failed to lower the mortality rate which appeared to be related to the chemotherapy.

Effect of CP and L-PAM on Tumor Growth. When 10 or 15 mg of L-PAM per kg were administered weekly to tumor-bearing mice, some tumor growth inhibition was noted (Table 3). The addition of CP to 10 mg of L-PAM per kg significantly enhanced the tumor growth-inhibiting effect of the chemotherapeutic agent. When 15 mg of L-PAM per kg were used with CP, death of almost all mice occurred by 6 weeks. The addition of CP failed to permit the use of a larger dose of L-PAM.
tumor growth was observed (Chart 4). The addition of CP to MTX significantly increased tumor inhibition. The increase, however, was not as great as that observed when CP was added to CY. As with other drugs, the addition of CP failed to decrease the mortality resulting from MTX.

DISCUSSION

This series of investigations has demonstrated that greater tumor growth inhibition was achieved when CP was used in combination with an alkylating agent (CY or L-PAM) than when used with an antimetabolite (5-FU). Greater inhibition occurred with CP + CY than with CP + L-PAM, suggesting that factors other than the general type of the drug may be responsible for these findings. In experiments in which 2 singly used chemotherapeutic agents resulted in a similar repression of tumor growth, i.e., CY and 5-FU or CY and L-PAM, the use of CP in combination with each of the agents had a different inhibitory effect. Moreover, the addition of CP to a combination of chemotherapeutic agents resulted in no greater tumor growth inhibition than that which occurred when CP was used along with the most effective single agent in the combination. This was despite the fact that the combination of drugs (without CP) was more effective in inhibiting tumor growth than were any of the components used individually, a finding in keeping with clinical observations regarding the use of combination chemotherapy.

Reasons for these findings must be speculative, but they could be related to 1 or several factors. The possibility may be considered that CP alters the cytostatic properties of certain of the chemotherapeutic agents and not others by...
differently affecting their metabolism. For example, it has been established that the effectiveness of CY is due to its activation by hepatic microsomal enzymes and that the active metabolites reach their target sites (tumor cells) by traversing the systemic circulation (2, 4, 21). A slow release of the active agent(s) from CY might result in more sustained blood and tissue levels of the active form of the drug and consequently a greater antitumor effectiveness. Since some evidence is available to suggest that CP may indeed be a microsomal inhibitor (7), it may thus advantageously interfere with the metabolism of CY. The results achieved by us using \[^{14}C\]CY have supplied evidence in support of this hypothesis (10).

On the other hand, since the metabolic pathways of 5-FU are entirely different from those of CY (3, 14, 17), they may be affected differently or not at all by CP. Activation of 5-FU occurs within target cells. It is within target cells that the drug is activated to nucleotides that alter RNA synthesis and function as well as ribosomal stability and interfere with thymidylate synthesis (3). No evidence exists to indicate that CP directly affects target cells and is thus likely to have no effect on the metabolism of 5-FU in contrast to CY. Whether or not CP alters the metabolic pathways of other drugs, including those used in these investigations (L-PAM or MTX), enough to influence to differing degrees their effect on tumor growth must remain a consideration.

A 2nd possibility that might account for these findings is that the various agents alone or in combination interfere differently with the effector mechanism(s) responsible for the action of CP. One theoretical objection to the use of combined chemo- and immunotherapy is related to interference of action of the immunotherapeutic agent by the cytotoxic agent. That such is at least not always the case has been convincingly shown by the results obtained after combining CY and CP (9, 11, 12) unless, of course, the entire effect of that combination is due to alteration of CY metabolism by the CP rather than to an immunostimulating property of the CP. Conceivably, other chemotherapeutic agents interfere with the effectors mechanisms responsible for the action of CP differently than does CY, thus accounting for the observed differences.

Although the precise mechanism(s) through which CP exerts its immunopotentiating effect remains to be elucidated, evidence suggests that its tumor-inhibiting properties are not dependent on the T-lymphocyte (23, 24). Consequently, while pyrimidine analogs have been shown to possess T-cell depressant properties (5, 6), the lesser effectiveness of 5-FU than CY with CP probably cannot be related to this attribute of the former (5-FU). While CY administration also results in a quantitative decrease in T-cells, it is a potent B-cell depressant (12, 22). Nonetheless, as has been repeatedly demonstrated in this laboratory, the use of CP, a B-cell-oriented immunopotentiator (15, 19), is most effective when administered with CY (9, 11, 12), suggesting that the tumor-inhibiting properties of the combination are not mediated by B-cells. However, the possibility must be considered that CP could be circumventing or preventing the effect of CY on B-lymphocytes by stimulating repopulation of these cells. To the best of our knowledge, there are no published data regarding the effect of L-PAM on the sub-populations of lymphocytes. While evidence is scant, it seems unlikely that the observed differences in tumor response to various chemotherapeutic agents when used with CP are related to differences in lymphocytes. There is increasing evidence that suggests an important role for the macrophage in mediating the antitumor properties of this immunopotentiator (1, 13, 18). A series of investigations by us are in progress to evaluate the effect of various chemotherapeutic agents on such cells. Early results indicate that CY actually increases in vitro bone marrow macrophage colony production (8).

Whatever the mechanism(s) responsible for these findings, the greatest significance of these observations is that they provide data that could be helpful in the design of clinical trials using CP. At present such trials using chemotherapeutic agents and immunotherapy are being formulated empirically without a data base to supply direction.

It has been suggested from clinical findings that CP may, by lessening drug toxicity, permit the administration of larger doses of chemotherapeutic agents (16). These present findings fail to substantiate such a clinical impression. In fact, they suggest that the use of CP increases the mortality rate as a result of the cytotoxic agents.

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The Effect of Corynebacterium parvum in Combination with 5-Fluorouracil, L-Phenylalanine Mustard, or Methotrexate on the Inhibition of Tumor Growth


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