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Effects of Dose and Schedule of Immune Stimulant on Efficacy of Combination *Corynebacterium parvum*-Cyclophosphamide Treatment for a Murine Mammary Adenocarcinoma[1]

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ABSTRACT

Certain variables which might influence the outcome of combining cytotoxic drug and immune stimulant therapy were studied to optimize the effectiveness of *Corynebacterium parvum* combined with cyclophosphamide (CY) as treatment for a murine mammary adenocarcinoma (CaD_2). Optimal effects of combined *C. parvum*-CY treatment in the CaD_2 system were obtained when 443 to 1400 μg of this immune stimulant per mouse were injected 2 to 3 days after CY chemotherapy and when combination treatment was continued on a weekly basis. The most critical factors contributing to the effectiveness of combination treatment in this system were the dose of *C. parvum* and the treatment frequency. The interval between chemotherapy and immune stimulant therapy was less critical to the outcome of combination treatment. Combination treatment given once or weekly significantly decreased tumor size in comparison to single or weekly CY treatment. A single treatment with CY and *C. parvum* significantly improved the survival over mice given a single CY treatment, but weekly CY and *C. parvum* treatment did not increase the survival over mice, given weekly chemotherapy.

INTRODUCTION

In a previous communication (7), we reported the effects of weekly systemic (i.p.) *Corynebacterium parvum* treatment in conjunction with weekly CY[4] treatment on the growth of the murine mammary adenocarcinoma CaD_2. Although systemically administered *C. parvum* or CY alone significantly retarded the growth of CaD_2 tumors, combination treatment was significantly more effective in controlling tumor growth than was either agent administered alone and in some cases resulted in tumor-free survivors. The results of that study prompted us to investigate certain additional variables which might influence the effectiveness of combination treatment. In this paper, we report the influence of: (*a*) the interval between CY treatment and *C. parvum* treatment; (*b*) the dose of *C. parvum* combined with a fixed amount of CY; and (*c*) the frequency of treatment with CY and *C. parvum* on the effectiveness of combination treatment.

MATERIALS AND METHODS

Mice. Female BALB/c × DBA/2J F_1 (hereafter called $CD2F_1$) mice were obtained from Charles River Breeding Laboratories through the auspices of the Drug Research and Development Branch, National Cancer Institute, Bethesda, Md.

Tumor. The spontaneous, poorly differentiated mammary adenocarcinoma CaD_2 was obtained from The Jackson Laboratory, Bar Harbor, Maine. The CaD_2 tumor was maintained in our laboratory by serial s.c. passage of tumor cell suspensions (10^6 cells/0.1 ml) in female $CD2F_1$ mice. Cell suspensions were prepared as described previously (6). Tumor cell concentrations for injection were determined by hemocytometer counts of trypan blue-excluding cells. Viability in most cases was above 95%.

Immune Stimulant. Suspensions of killed *C. parvum* (CN6134) which contained 7 mg (dry weight) killed bacteria per ml were provided by Burroughs Wellcome Co., Research Triangle Park, N. C. Dilutions were made in sterile 0.9% NaCl solution.

Cytotoxic Drug. CY powder was purchased from Mead Johnson & Co., Evansville, Ind. The stock solution and dilutions were prepared in 0.9% NaCl solution immediately prior to use.

Tumor Mensuration. The length and width of tumors were measured 2 to 3 times/week with calipers, and sizes were expressed as the product of the diameters in sq mm ± S.E. In the tables, data are reported for tumor size on Days 20 or 21. This was done because most (80% or more) of the animals in the control and experimental groups were alive at those times. The effects seen on Days 20 or 21 are representative of those seen at other times.

Statistical Tests. One-way analysis of variance was used to test whether differences in size of tumors in different groups of an experiment were accounted for by chance. When significance was found, the Newman-Keuls test (9) was used to identify which experimental subgroups differed significantly from each other. Differences in survival time were assessed by the Mann-Whitney *U* test.

RESULTS

The optimal interval for combining *C. parvum* with CY was determined in the first experiment. One hundred twenty

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[4] The abbreviation used is: CY, cyclophosphamide.

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mice received 5 × 10^4 CaD_2 cells s.c. and were given either no treatment, a single i.p. injection of CY (45 mg/kg) 3 days after tumor cell inoculation, a single i.p. injection of *C. parvum* (1400 μg/mouse) at different times after tumor cell injection, or a single injection of CY (45 mg/kg) 3 days after tumor cell injection combined with a single injection of *C. parvum* (1400 μg/mouse) 1 to 9 days after CY. The results recorded in Table 1 show that all single-agent treatments significantly inhibited tumor growth, in comparison to results in untreated mice. *C. parvum* administered on Days 4, 5, 6, or 7 after tumor cell inoculation produced nearly equivalent antitumor effects, but *C. parvum* given after 12 days of tumor growth was less effective. Combination therapy was significantly more tumor inhibitory than was treatment with CY or *C. parvum* alone. Strong and nearly equivalent tumor inhibition was obtained when *C. parvum* was administered either 1, 2, 3, or 4 days after CY, but *C. parvum* administered 9 days after chemotherapy had no significant effect.

The efficacy of different amounts of *C. parvum* combined with the standard dose of CY was studied in the next experiment. One hundred forty mice received 5 × 10^4 CaD_2 cells s.c. and were given either no treatment, a single i.p. injection of CY (45 mg/kg) 3 days after tumor cell injection, a single i.p. injection of *C. parvum* (4.3 to 1400 μg/mouse) 6 days after tumor cells, or a single i.p. injection of CY (45 mg/kg) on Day 3 after tumor cells combined with a single i.p. injection of different amounts of *C. parvum* 3 days after chemotherapy. The results (Table 2) indicate that *C. parvum* in amounts of 1400 and 443 μg/mouse significantly decreased tumor growth and prolonged survival, compared to results in untreated controls. Mice treated with 140 μg *C. parvum* had tumor sizes similar to untreated mice, but the survival of these mice was prolonged relative to untreated mice. We have no explanation for this anomalous result.

Table 1
Optimal interval for combining C. parvum with CY as treatment for CaD_2 tumors

Day of treatment[a]			
CY	*C. parvum*	*N*	Tumor size (sq mm) on Day 21
–[b]	–	8	274 ± 9[c]
3	–	9	174 ± 17[d]
–	4	10	138 ± 10[d]
–	5	9	156 ± 11[d]
–	6	10	143 ± 11[d]
–	7	9	155 ± 23[d]
–	12	8	209 ± 15[d]
3	4	10	93 ± 16[e]
3	5	10	68 ± 7[e]
3	6	10	67 ± 7[e]
3	7	10	83 ± 16[e]
3	12	9	133 ± 14

[a] All mice received 5 × 10^4 CaD_2 cells s.c. on Day 0 and were given either no treatment, a single injection of CY (45 mg/kg i.p.) on Day 3, a single injection of *C. parvum* (1400 μg/mouse i.p) on the days indicated, or a combination of the 2 drugs on the days indicated.
[b] –, no treatment.
[c] Mean ± S.E.
[d] $p < 0.05$ compared to untreated controls.
[e] $p < 0.05$ compared to CY-treated controls or those given *C. parvum* alone on the same day.

Table 2
Effect of a dose of C. parvum combined with CY as treatment for CaD_2 tumors

Treatment[a]				
Day 3 (mg CY/kg)	Day 6 (μg *C. parvum*/mouse)	*N*	Tumor size (sq mm) on Day 21	Median survival time (days)
–[b]	–	12	269 ± 44[c]	28
–	4.3	7	182 ± 23	28
–	14	8	204 ± 24	37
–	44	9	193 ± 43	34
–	140	9	243 ± 46	42[f]
–	443	10	136 ± 34[d]	38[f]
–	1400	9	110 ± 21[d]	36[f]
45	–	11	176 ± 18	34
45	4.3	9	145 ± 43	35[f]
45	14	10	100 ± 13[d, e]	35[f]
45	44	10	215 ± 45	30
45	140	11	97 ± 12[d, e]	38[f]
45	443	9	41 ± 9[d, e]	43[f]
45	1400	10	52 ± 3[d, e]	36[f]

[a] All mice received 5 × 10^4 CaD_2 cells s.c. on Day 0 and were given either no treatment, a single injection of CY (45 mg/kg i.p.), a single injection of *C. parvum* (μg per mouse i.p.) as indicated, or a combination of the 2 drugs as indicated.
[b] –, no treatment.
[c] Mean ± S.E.
[d] $p < 0.05$ compared to untreated controls.
[e] $p < 0.05$ compared to CY-treated controls.
[f] $p < 0.05$ compared to untreated controls; $p > 0.05$ compared to CY- or *C. parvum*-treated controls.

Treatment with other amounts of *C. parvum* was ineffective, as was treatment with CY.

When combined with CY, all amounts of *C. parvum* with the exception of 4.3 and 44 μg/mouse significantly decreased tumor size, in comparison to results in untreated mice or mice treated with CY. All doses of *C. parvum*, with the exception of 44 μg/mouse, combined with CY improved survival, compared to results in untreated mice. However, no combination treatment improved survival over that obtained by treatment with CY or *C. parvum* alone. With 2 exceptions (140 μg *C. parvum* alone and CY combined with 4.3 μg *C. parvum*), treatments which prolonged survival also significantly decreased tumor size.

We next studied the relative effectiveness of combination therapy when given once or repeatedly. Eighty mice received 5 × 10^6 CaD_2 cells s.c. and were given either no treatment, a single i.p. injection of CY (45 mg/kg) 3 days after tumor cell injection, weekly i.p. injections of CY beginning on Day 3 after tumor cells, a single i.p. injection of *C. parvum* (1400 μg/mouse) 6 days after tumor cells, weekly i.p. injections of *C. parvum* starting 6 days after tumor cells, or single or weekly treatment with both agents. Table 3 shows that, with the exception of single CY treatment, all single-agent treatments significantly retarded tumor growth compared to results in untreated mice. Weekly CY treatment was significantly more effective than was treatment given only once, but weekly *C. parvum* treatment was not more effective than was a single treatment with this agent. Combination therapy, given either once or on a weekly basis, was significantly more tumor inhibitory than was treatment with CY alone. Weekly combination treatment resulted in significantly smaller tumors than did com-

Table 3

Effect of single or repeated treatment with C. parvum combined with CY on the growth of CaD_2 tumors

Treatment[a]					
Day 3	Day 6	Frequency	N	Tumor size (sq mm) on Day 20	Median survival time (days)
-[b]	-	-	9	210 ± 12[c]	28
-	*C. parvum*	Single	10	82 ± 12[d]	38[g]
CY	-	Single	8	143 ± 13	31
CY	*C. parvum*	Single	15	39 ± 8[d, e]	40[g, h]
-	*C. parvum*	Weekly	10	73 ± 11[d]	33
CY	-	Weekly	10	54 ± 10[d]	44[g]
CY	*C. parvum*	Weekly	15	14 ± 3[d-f]	52[g, i]

[a] All mice received 5 × 10^4 CaD_2 cells s.c. on Day 0 and were given either no treatment or single or weekly injections of CY (45 mg/kg i.p.), *C. parvum* (1400 μg/mouse i.p.), or a combination of the 2 drugs.
[b] -, no treatment.
[c] Mean ± S.E.
[d] $p < 0.05$ compared to untreated mice.
[e] $p < 0.05$ compared to corresponding CY-treated controls.
[f] $p < 0.05$ compared to mice given single combined treatment.
[g] $p < 0.05$ compared to untreated mice.
[h] $p < 0.05$ compared to mice given single CY treatment.
[i] $p > 0.05$ compared to mice given weekly CY treatment; $p < 0.05$ compared to mice given single combined treatment.

bination treatment given only once. All treatments with the exception of CY given once and weekly *C. parvum* treatment significantly improved survival of tumor-bearing mice. The greatest increase in survival was obtained with weekly combination treatment, but the increase was not significant compared to that for mice given only weekly CY treatment. Combination treatment given once only did, however, significantly improve survival compared to treatment with a single dose of CY. With one exception (weekly *C. parvum* treatment), all treatments which significantly decreased tumor size relative to results in untreated controls also significantly increased survival of the tumor-bearing mice.

On the basis of the preceding 2 experiments, we determined the relative effectiveness of different doses of *C. parvum* used in weekly combination treatment with CY. One hundred five mice received 5 × 10^4 CaD_2 cells s.c. and were given either no treatment, weekly i.p. injections of CY (45 mg/kg) beginning 3 days after tumor cells, weekly i.p. injections of *C. parvum* (1400, 433, or 14 μg/mouse) starting 6 days after tumor cell inoculation, or weekly i.p. injections of CY beginning on Day 3 after tumor cells combined with weekly i.p. injections of *C. parvum* (1400, 443, or 14 μg/mouse) beginning 6 days after tumor cells. The results shown in Table 4 indicate that all single-agent treatments significantly inhibited tumor growth with the exception of *C. parvum* at a dose of 14 μg/mouse. The effect of *C. parvum* administered weekly at a dose of 1400 μg/mouse was roughly equivalent to that obtained by weekly administration of CY. *C. parvum* administered at a dose of 443 μg/mouse was significantly more tumor inhibitory than it was when given at a dose of 14 μg/mouse. Combination therapy produced significantly better tumor inhibition than did either agent given alone. Tumor control obtained by treatment with CY combined with *C. parvum* (443 μg/mouse) was equal to that obtained with CY combined with *C. parvum* (1400 μg/mouse), but the degree of tumor control obtained with 14 μg *C. parvum* combined with CY was significantly less effective. Combination therapy significantly increased survival of tumor-bearing mice, whereas treatment with *C. parvum* or CY alone did not significantly increase survival, although the latter approached significance.

The effect of *C. parvum* treatment alone on survival in this experiment, as well as in the other experiments reported here, is worthy of comment. *C. parvum* in an amount ≥ 443 μg/mouse (single or weekly treatment) consistently retarded tumor growth, but it had no consistent effect on survival. Weekly treatment with *C. parvum* was not better than a single treatment in improving survival and in some cases was less effective than was single treatment with *C. parvum*. Similarly, lower doses of *C. parvum* sometimes had a greater effect on survival than had higher doses of this immune stimulant.

In these experiments, no treatment, single agent, or combined agent resulted in tumor-free survivors; *i.e.*, although survival was improved in some cases, all mice eventually died of their tumors.

DISCUSSION

We have studied certain variables which might influence the outcome of combination therapy to optimize the effectiveness of *C. parvum* and CY as treatment for the CaD_2 mammary adenocarcinoma.

Table 4

Effect of weekly treatment with CY combined with different amounts of C. parvum on the growth of CaD_2 tumors

Weekly treatment[a] begun				
Day 3	Day 6	N	Tumor size (sq mm) on Day 21	Median survival time (days)
-[b]	-	10	222 ± 9[c]	31
-	*C. parvum*, 14 μg/mouse	10	217 ± 7	37
-	*C. parvum*, 443 μg/mouse	10	98 ± 9[d]	35
-	*C. parvum*, 1400 μg/mouse	10	75 ± 11[d]	40
CY	-	8	76 ± 9[d]	46[f]
CY	*C. parvum*, 14 μg/mouse	15	39 ± 6[e]	52[g]
CY	*C. parvum*, 443 μg/mouse	15	13 ± 3[e]	59[g]
CY	*C. parvum*, 1400 μg/mouse	15	18 ± 3[e]	47[g]

[a] All mice received 5 × 10^4 CaD_2 cells s.c. on Day 0 and were given either no treatment, CY (45 mg/kg i.p.), *C. parvum* (μg/mouse i.p.) as indicated, or a combination of the 2 drugs.
[b] -, no treatment.
[c] Mean ± S.E.
[d] $p < 0.05$ compared to untreated mice.
[e] $p < 0.05$ compared to CY-treated mice.
[f] $p \simeq 0.05$ compared to untreated controls.
[g] $p < 0.05$ compared to untreated controls; $p > 0.05$ compared to CY-treated controls; $p < 0.05$ compared to *C. parvum*-treated controls.

Our results indicate that the antitumor activity of a single i.p. injection of CY (45 mg/kg) could be significantly augmented by a single i.p. injection of *C. parvum* (1400 μg/mouse) given 1 to 4 days after chemotherapy and that *C. parvum* injected 2 or 3 days after CY was most effective in augmenting the effects of chemotherapy. *C. parvum* injected 9 days after CY did not augment chemotherapy.

Our earlier study (7) showed that significant improvement of CY chemotherapy could also be obtained by administering *C. parvum* 3 days prior to CY or at the same time as CY. Those results together with our present findings indicate a broad effective schedule for combining *C. parvum* with CY.

The present study is at odds with our earlier study (7) in relation to cures obtained by combination treatment; *i.e.*, we observed no tumor-free survivors in the present study, whereas we reported (7) cure rates of 7 of 20 (35%) and 9 of 15 (60%) after weekly treatment with CY and *C. parvum* administered in the reverse order as used here. We have no explanation for this discrepancy. Possibly, the difference in sequence of chemotherapy and immune stimulant therapy accounts for this difference, but in other experiments tumor-free survivors have been obtained with either sequence and also by simultaneous treatment with CY and *C. parvum* (Ref. 7; unpublished results).

In relation to the dose of *C. parvum* used in conjunction with CY, strong improvement in the chemotherapeutic effect of CY was obtained with doses of *C. parvum* ranging from 140 to 1400 μg/mouse. Within this range, doses of 443 and 1400 μg/mouse were optimal. Significant augmentation of CY chemotherapy was also obtained with *C. parvum* (14 μg/mouse), but doses of 44.3 and 4.3 μg/mouse were ineffective.

These results indicate that the dose of *C. parvum* which is administered with CY is a more critical variable in relation to the effectiveness of combination treatment than is the interval between chemotherapy and immune stimulant.

Also of importance to the degree of tumor control achievable with combination treatment as well as single-agent treatment was the treatment frequency. Weekly CY treatment significantly decreased tumor size and also significantly increased survival time, in comparison to results in mice given no treatment. A single CY treatment had no significant effect on either.

Single or weekly *C. parvum* treatment significantly decreased tumor size compared to results in untreated mice and were equivalent in this regard. In contrast, only single *C. parvum* treatment significantly improved survival compared to results in untreated mice. A single treatment with CY and *C. parvum* significantly decreased tumor size compared to results in untreated mice and mice given a single CY treatment; it also improved survival compared to results in untreated mice but not to results in CY-treated mice. Weekly treatment with CY and *C. parvum* significantly decreased tumor size in comparison to results in untreated mice, mice given weekly CY treatment, and mice treated only once with CY and *C. parvum*. This therapeutic schedule significantly increased survival compared to results in untreated mice and mice given single treatment with CY and *C. parvum* but not compared to results in mice given weekly CY treatment.

When different doses of *C. parvum* were combined with CY as weekly combination treatment for the CaD_2 tumor, it was found that a dose as low as 14 μg/mouse, which by itself had no effect on tumor growth, significantly augmented the effects of CY. This could be taken as evidence for a true synergism of CY and *C. parvum*. Higher doses of *C. parvum* (443 and 1400 μg/mouse) produced a greater improvement in CY chemotherapy as indicated by effect on tumor size; however, for prolonging survival of tumor-bearing mice, lower doses of 443 and 14 μg/mouse were sometimes more effective than was a higher dose of *C. parvum*.

In these experiments, the optimal effects of combined *C. parvum* and CY treatment in the CaD_2 mammary adenocarcinoma system were obtained when 443 or 1400 μg of this immune stimulant per mouse were injected 2 to 3 days after chemotherapy and when combination treatment was continued on a weekly basis. Under these conditions, both tumor growth control and survival of tumor-bearing mice were maximized, compared to results in untreated mice. Compared to results in mice receiving weekly CY treatment, better control of tumor growth was obtained by combination treatment; paradoxically, weekly combination treatment failed to improve the survival of mice receiving only weekly chemotherapy.

Our results are similar to those obtained by Fisher *et al.* (4), but they differ from those of other investigators who have reported that the interval between administration of chemotherapy and immune stimulant therapy was critical to the effectiveness of the combination treatment (1, 5).

In conclusion, the mechanism(s) which underlies the effect of *C. parvum* on CY chemotherapy is unknown. It could be related to an immunomodulatory effect of *C. parvum* which results in an enhanced ability of the immune system to attack tumor cells, which in turn adds to the cytotoxicity of chemotherapy, but other mechanisms are also possible. For example, the cytocidal activity of CY requires that it be metabolized by microsomal mixed-function oxidases (8), and *C. parvum* has been reported to inhibit the function of such enzymes (2). Fisher *et al.* (3) obtained evidence in a C3H tumor system that systemically administered *C. parvum* decreases the metabolism of CY and suggested that this may account, at least in part, for the synergism of these agents when used in combination. This explanation could also be true in our tumor system, but we observed significant augmentation of the effect of a single injection of CY by a single injection of *C. parvum* given 1 to 4 days after CY, *i.e.*, at times when no CY would remain in the animal. This result suggests that the effects of *C. parvum* on CY chemotherapy must include some mechanism(s) other than an effect on CY metabolism.

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