[CANCER RESEARCH, 36, 2241-2247, July 1976]

Correlation of Antitumor Chemoimmunotherapy with Bone Marrow Macrophage Precursor Cell Stimulation and Macrophage Cytotoxicity[1]

Bernard Fisher and Norman Wolmark

With the technical assistance of Jean Coyle, Morton Levine, and Elizabeth Saffer

Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

SUMMARY

The present investigations have assessed the effects of prolonged cyclophosphamide (CY) and *Corynebacterium* (CP) treatment on the production of bone marrow macrophage precursors [colony-forming cells (CFC)] and on the cytotoxicity of macrophages comprising colonies produced by the CFC. The findings have been correlated with tumor growth in animals receiving the immunochemotherapy. In addition, studies have been directed toward ascertaining whether the administration of CP with CY might lessen the myelosuppressive effects of the latter. Following each consecutive weekly dose of CY (even after as many as 11), there was a significant depression in the number of bone marrow cells (BMC's) but, by the next injection, marrow cellularity had returned to normal. When the number of BMC's was reduced, the proportion of the remaining cells, which consisted of CFC, was increased. Upon reconstitution of the marrow, the proportion of CFC returned to the level of the controls. The total number of CFC in marrow was at no time following CY therapy significantly less than the number in marrow of untreated mice. The addition of CP to the treatment regimen with CY resulted in an absolute as well as relative increase in CFC at all times during administration of the combined therapy, *i.e.*, when there was a depression in total numbers of marrow cells, as well as when marrow restoration had occurred. Although CP stimulated the number of cells entering into differentiation, it failed to affect the total BMC population when given during CY therapy. Numbers of BMC's had been neither increased nor prevented from decreasing, by CP administration, indicating that the use of total cellularity as an index of the CP marrow-sparing effect is without merit. The present results relative to cytotoxicity of macrophages derived from the CFC concur with and extend our previous findings indicating that the cytotoxic property of macrophages originates in its ancestral stem cell or CFC and that factors responsible for increasing the CFC population do not selectively stimulate precursor cells responsible for production of the cytotoxic macrophage. Although the proportion of cytotoxic macrophages was not altered by CP when administered with CY, the absolute number of such cells was increased. Since the increase in macrophage colony production and, consequently, in cytotoxic macrophages correlates with increased inhibition of tumor growth when CP was used with CY, it is suggested that macrophage precursors are the cells of primacy in CP immunopotentiation. Their stimulation, resulting in enhanced cytotoxic macrophage formation, could be responsible for the inhibition of tumor growth observed in our model system. The findings also suggest that when myelosuppression is a limiting factor in the use of a chemotherapeutic agent, the concomitant use of CP may be advantageous.

INTRODUCTION

Studies from this laboratory (10, 11) have shown that arrest of growth, as well as partial and complete regression of established C3H mammary tumors, occurred consistently following the asynchronous administration of CY[2] and CP. Inhibition of growth was always greater than that resulting from the use of either agent alone. The model system used has provided a unique opportunity to identify alterations in a spectrum of host responses resulting from the therapy and to relate them to the changes observed in tumor growth. Such a correlation could permit a more precise identification of factors responsible for tumor growth inhibition and the subsequent formulation of better treatment regimens. While such investigations were being carried out, attention was directed toward the macrophage. Increasing evidence indicates involvement of that cell in the response of a host to its tumor (5, 6, 16, 18) and in the potentiation of the immune response by CP (1, 13, 17, 20, 21). Our own studies in that regard have revealed that there was a significant, although transient, increase in the number of CFC in bone marrow of C3H mice upon implantation of a syngeneic mammary tumor (2). With appropriate culture of BMC's, the CFC, derived from marrow stem cells, produce colonies of mature macrophages. Enhanced colony production is indicative of an increased proportion of CFC in bone marrow. Macrophages comprising such colonies have been found by us (8) to be specifically cytotoxic to cells from the immunizing tumor. They maintained that characteristic as long as a tumor was present in the animal from which the bone

[1] Supported by USPHS Grants CA 14972 and CA 13289.
Received September 30, 1975; accepted April 5, 1976.

[2] The abbreviations used are: CY, cyclophosphamide; CP, *Corynebacterium parvum;* BMC, bone marrow cell; CFC, colony forming cell.

marrow was derived. It has also been observed that the number of CFC (macrophage colonies) was enhanced when normal (21) and tumor-bearing (20) mice were administered CP, indicating stimulation of CFC production by that immunopotentiator.

Administration of CY has been shown to result in a profound decrease in marrow cellularity followed by a rapid recovery (12, 15, 19). Hematopoietic stem cells are particularly vulnerable to destruction. Aside from studies assessing hematopoietic stem cell kinetics in normal animals after a single dose of CY, little or no other data are available concerning those cells. More specifically, information from tumor-bearing animals in regard to the effect of either a single dose or the prolonged administration of CY on CFC production is lacking, as is that relative to the cytotoxicity of macrophages generated by the CFC.

The present investigations were primarily carried out to assess the effect of prolonged CY administration with and without CP (as used in our model system) on the production of CFC, as well as on the cytotoxic properties of macrophages comprising the colonies produced by the CFC, and to correlate the findings obtained with growth of tumors in animals receiving that immunochemotherapy. In addition, since CP stimulates proliferation of hematopoietic precursor cells, it was considered appropriate to ascertain whether the administration of CP with CY might diminish the myelosuppressive effects of the latter. Inasmuch as immunochemotherapeutic regimens have been proposed for treating human tumors, such information would have practical importance. It has been proposed (14) that patients receiving CP may tolerate larger doses of a chemotherapeutic agent because of less myelosuppression.

MATERIALS AND METHODS

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

Tumor. The tumor used was a mammary carcinoma arising in a C3H female and carried in transfer in C3HeB mice. In all experiments, tumors were transferred by inoculating a suspension of 2×10^5 viable tumor cells in 0.1 ml Medium 199 s.c. into the left hind leg distal to the popliteal node. A tumor approximately 5 mm in diameter developed by Day 14, at which time treatment was begun. 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 were 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 CN6134 (supplied by Dr. J. D. Whisnant of Burroughs-Wellcome Company, Research Triangle Park, N. C.) was administered i.p. in a dose of 1.4 mg, dry weight, of organisms every 7 days.

CY. The CY was dissolved in distilled water so that the desired dose was contained in 0.01 ml/g body weight. The dose of CY used was 90 mg/kg body weight. All injections of this agent were given i.p. every 7 days.

Combination Therapy. When weekly CP was used in combination with weekly CY, it was given 4 days following the CY. In this paper, the time between administration of chemotherapy (CY) and immunotherapy (CP) is referred to as the C-I interval. Animals in each treatment group were randomly distributed so that femurs were obtained from 3 mice in each group 4 days after each dose of CY (day of CP treatment) or 7 days after CY (day of next CY treatment).

Bone Marrow Macrophage Assay. A modification of the method of Bradley and Metcalf (3) used by us in previous studies and described in detail elsewhere (2) was employed. No colony-stimulating factor was utilized. All animals were killed by cervical dislocation, immersed in antiseptic solution, and 1 femur was promptly removed by sterile technique from each mouse. The marrow cell suspension was counted and diluted to contain 1×10^6 cells/ml. Then 1.5 ml of the cell suspension were added to 13.5 ml Medium 1066 containing 1.8% methylcellulose and 15% horse serum. One ml of the resulting suspension (containing 1×10^5 cells) was plated in each of 10 tissue culture dishes (35 × 10 mm; Falcon Plastics Company, Oxnard, Calif.). Plates were incubated at 37° in a 10% CO_2 atmosphere with 100% humidity for 7 days. After that period of incubation, discrete colonies of 25 or more cells, as well as small clusters of fewer cells, were found. The cells were morphologically similar to macrophages and, in addition, adhered to glass and phagocytized India ink particles. Identification marks on the plates were replaced by a code to ensure objectivity of the colony counts. Only groups of 25 or more cells arranged in a colony configuration were counted. All plates in an experiment were counted by 1 observer, but the results obtained by 3 different observers were in good agreement.

Bone Marrow Macrophage Cytotoxicity Assay. To produce large numbers of macrophages for cytotoxicity testing, a colony-stimulating factor was added to the culture medium. Diluted BMC suspensions, obtained as described above, were added to an equal volume of mouse L-cell (NCTC clone 929) -conditioned medium and 4 volumes of Medium 1066 containing 1.8% methylcellulose and 15% horse serum. The material was mixed for 15 min with a magnetic stirring bar, and 40-ml aliquots were placed in 250-ml Falcon flasks. The flasks were incubated for 7 days in a 10% CO_2 100% humidity atmosphere. At that time, cells harvested were, but for a rare exception, macrophages. The morphology of these cells is currently being studied by electron microscopy to determine whether differences exist between the cells produced in culture from the bone marrow of tumor-bearing and normal mice. The macrophages were isolated from the culture medium by washing 4 times with 0.9% NaCl solution and resuspended in Earle's minimal essential medium with 30% fetal calf serum. The final suspension of macrophages contained 95 to 100% viable cells by the trypan blue exclusion test.

The cytotoxicity of the macrophages was assayed by the method previously described for lymph node cells (7). The cells to be tested were added to wells in microtest plates which were covered by a monolayer of tumor cells. Each plate included wells with no added cells, macrophages from nontumorous mice, and regional lymph node cells from tumor-bearing mice, as well as the macrophages to be assayed. Plates were incubated for 48 hr, stained with crys-

tal violet, and examined. The wells were scored on a scale of 0 to 5 according to the area free of tumor cells, zero indicating complete coverage and 5 indicating complete absence of tumor cells. The data are presented both as the index of tumor cell destruction, representing the mean ± S.E. of at least 30 wells, and the percentage of tumor cell destruction. The latter compares the amount of tumor remaining in treated wells to that in nontreated control wells.

Statistical Analysis. Student's t test was applied to colony formation and cytotoxicity of macrophages. Comparisons of results between treatment groups were made only when all aspects of the experiment were identical relative to age of tumor and duration of treatment and BMC's were simultaneously cultured or assayed for cytotoxicity. Numbers of bone marrow cells per femur in 2 treatment groups were compared by means of paired comparison testing.

RESULTS

Effect of CY. Macrophage colony formation by 1×10^5 BMC's obtained from mice 4 days after CY treatment was increased over that produced by such cells from 0.9% NaCl solution-treated animals. By 7 days after CY inoculation, colony production had decreased so that it approximated that observed when BMC's were derived from 0.9% NaCl solution controls. Each weekly dose of CY produced the same cyclic pattern of colony formation, *i.e.*, a significant increase 4 days later and a return to the control level by Day 7. This was observed (Table 1) throughout an 8-week treatment period.

The number of BMC's obtained from femurs of CY-treated mice demonstrated an inverse relationship to the macrophage colony production (Table 1). At 4 days, the number of BMC's in CY-treated animals was lower than in the 0.9% NaCl solution-treated controls. By 7 days, the values were approximately the same in both groups. As a result of the inverse relationship between colony production and number of BMC's per femur, the number of colonies per femur was not significantly altered by CY, but, in most instances, the value was lower than in the controls. The cytotoxicity of the macrophages obtained from cultured BMC's was not affected by the administration of CY. As many as 12 weekly injections failed to diminish that property of the cells (Table 2).

Effect of CP and CY. Treatment of mice with CP in addition to CY produced a similar cyclic pattern of macrophage colony formation and of number of BMC's per femur, as was observed when animals were treated with CY alone (Table 3). However, whereas, following CY treatment alone, the number of macrophage colonies per plate of the treated group approximated that of the 0.9% NaCl solution controls at 7 days following drug administration, the addition of CP to the treatment resulted in a level of macrophage colony production that remained greater than that of 0.9% NaCl solution controls at that time. In addition, when CP was used, the number of colonies per femur was consistently greater than that of the 0.9% NaCl solution controls, in contrast to that observed when only CY was administered.

When direct comparisons were made between the 2 treatment groups (CY *versus* CP + CY), there was a reduction in number of BMC's/femur 4 days after CY followed by a recovery. At the various times of treatment, both reduction and recovery were practically equivalent in the 2 groups ($p > 0.05$) (Table 4). Macrophage colonies per plate or macrophage colonies per femur were always greater when CP was administered with CY, and the difference was almost always statistically significant. The addition of CP to CY was without effect relative to alteration of cytotoxicity of the macrophages derived from CFC (Table 2).

DISCUSSION

These studies have demonstrated that each weekly sublethal dose of CY given to a tumor-bearing animal resulted in

Table 1

Effect of CY on macrophage colony production by BMC's from tumor-bearing mice

Dose of CY[a]	Days of treatment	No. of experiments	No. of BMC's/femur (× 10^6)		No. of colonies/plate (1×10^5 BMC's)			No. of colonies/femur (× 10^3)		
			0.9% NaCl solution	CY	0.9% NaCl solution (A)	CY (B)	% (B/A)	0.9% NaCl solution (C)	CY (D)	% (D/C)
1	0									
	4	4	9.1 ± 0.63[b]	4.8 ± 0.29	89.5 ± 7.21[c]	234.3 ± 23.16	261[d]	8.5 ± 0.80	11.6 ± 1.31	136
2	7	4	10.9 ± 0.17	9.7 ± 0.59	67.0 ± 6.51	68.9 ± 5.23	102	7.4 ± 0.75	6.9 ± 0.49	93
	11	2	9.0	6.5	51.2 ± 3.26	96.1 ± 5.04	187[d]	5.2 ± 0.32	4.7 ± 0.35	91
3	14	4	10.4 ± 0.84	11.5 ± 1.44	81.5 ± 7.53	53.2 ± 5.19	65	8.7 ± 0.82	6.5 ± 0.76	75
	18	4	10.6 ± 0.74	4.3 ± 0.63	38.6 ± 4.18	117.8 ± 21.02	305[d]	4.4 ± 0.34	4.1 ± 0.50	94
4	21	6	12.0 ± 0.45	8.1 ± 1.67	52.0 ± 3.38	67.1 ± 5.45	129	6.3 ± 0.42	6.5 ± 0.43	104
	25	2	10.6	5.6	53.6 ± 2.36	111.1 ± 8.84	207[d]	6.1 ± 0.55	5.2 ± 0.29	84
7	42	2	14.9	14.9	21.1 ± 2.67	15.6 ± 1.19	74	3.1 ± 0.39	2.5 ± 0.19	83
	46	2	11.9	3.9	45.5 ± 4.62	79.7 ± 5.32	175[d]	6.7 ± 0.68	6.6 ± 0.44	98
8	49	2	14.1	12.5	49.3 ± 4.99	51.1 ± 2.73	103	6.5 ± 0.47	6.3 ± 0.36	97
	53	3	11.0 ± 0.83	7.1 ± 0.90	54.8 ± 2.83	86.3 ± 5.77	157[d]	6.2 ± 0.43	7.7 ± 0.97	123

[a] CY, 90 mg/kg, every 7 days.
[b] Mean ± S.E.
[c] Mean ± S.E. of total plates; 10/experiment.
[d] $p < 0.001$.

Table 2

Effect of CP and/or CY on macrophage cytotoxicity

Each time interval provides results of 1 experiment.

Days of treatment	Normal control	Tumor control	CY	CY + CP
4	0.40 ± 0.04[a] (4.8)[b]	3.65 ± 0.09 (72.0)	4.42 ± 0.09 (88.0)	
7	0.18 ± 0.07 (1.1)	3.25 ± 0.08 (64.1)	3.43 ± 0.09 (67.8)	3.37 ± 0.09 (66.6)
11	0.15 ± 0.07 (0.5)	3.15 ± 0.06 (62.1)	3.30 ± 0.08 (65.1)	3.73 ± 0.08 (74.0)
14	0.20 ± 0.07 (2.1)	3.30 ± 0.08 (65.4)	3.40 ± 0.09 (67.4)	3.58 ± 0.11 (71.1)
42	0.28 ± 0.08 (0)	3.72 ± 0.09 (72.9)	3.59 ± 0.10 (70.2)	3.62 ± 0.13 (70.8)
63	0.17 ± 0.04 (3.3)	No survivors	3.10 ± 0.10 (60.4)	3.15 ± 0.09 (61.5)
95	0.30 ± 0.08 (5.1)	No survivors	2.80 ± 0.12 (55.6)	3.00 ± 0.12 (59.6)
98	0.15 ± 0.06 (2.9)	No survivors	3.15 ± 0.10 (62.0)	2.83 ± 0.14 (55.4)

[a] Index of tumor cell destruction; mean ± S.E. of 30 wells.
[b] Numbers in parentheses, percentage of tumor cell destruction.

a significant suppression in the number of BMC's. By the time of the next injection, however, marrow cellularity had returned to approximately its pretreatment level. That cyclic pattern persisted unchanged even after as many as 11 consecutive weekly treatments. These findings have added to those of others (4) which have indicated that, following a single dose of CY, myelosuppression was most marked about 4 days after therapy and that, subsequently, the bone marrow was rapidly reconstituted. At a time (4 days after each dose of CY) when the total number of BMC's was reduced, the proportion of remaining cells that consisted of CFC was significantly increased over that found in the marrow of normal mice. When the marrow population became reconstituted, the proportion of CFC once again approximated that in nontreated controls. Thus, the total number of CFC in the marrow was at no time following CY therapy significantly less than the number in marrow of untreated mice. The supranormal proportion of CFC observed 4 days after treatment coincides with the findings of Fried and Johnson (12) who noted that the "endocolonizing" potential of hematopoietic stem cells, *i.e.*, their ability to colonize in the spleen of the same animal after lethal X-irradiation with a portion of the body shielded, was enhanced 4 days after a single injection of CY.

Several explanations for the findings occurring after CY therapy may be considered. That there was a preferential sparing of CFC by CY seems unlikely, since the primary effect of the hematopoietic insult is on stem cells rather than on those that have differentiated. That the increased proportion of CFC was the result of a more rapid migration of the mature elements of the marrow to repopulate peripheral sites depleted by the CY cannot be totally discounted. It is more likely, however, that there occurred an acceleration in the rate of replication of surviving hematopoietic stem cells with a concomitant increase in CFC.

The observation that there was a significantly increased number of CFC when CP was added to the treatment regimen with CY is a salient finding of these investigations and requires consideration, particularly in relation to the synergistic inhibition of tumor growth resulting from administration of both agents. Not only was there an increase in the proportion of CFC at a time when there was a depression in the total number of marrow cells as a result of the CY, but this was also evident when marrow restoration, *i.e.*, total numbers of cells, had returned to normal levels. The finding that the number of colonies per femur was greater at all times during the combined CP and CY therapy than during CY therapy alone indicates that the increase in CFC due to CP was not only a relative one, but was absolute as well.

Recently, we reported (8) that macrophages, which are constituents of the colonies formed when bone marrow cells (CFC) are cultured in semisolid medium, are specifically cytotoxic to cells from the immunizing tumor. The findings have indicated that the cytotoxicity of the macrophage originates in its ancestral stem cell or CFC. It is those cells which become "programmed" to transmit that quality to macrophages. Moreover, it has been postulated that receptor sites of the CFC (or stem cells) that respond to a stimulus for self-replication are probably different from those sites that, when activated, result in cytotoxic properties of their progeny. The present findings concur with those conclusions, for they have indicated that the proportion (percentage) of cytotoxicity remained relatively constant, despite the variations in numbers of CFC present, as a result of therapy with CP and/or CY. Such findings suggest that factors responsible for increasing the CFC population do not selectively stimulate precursor cells responsible for production of the cytotoxic macrophage. They provide further evidence indicating that macrophage colony production and macrophage cytotoxicity are the result of separate mechanisms.

These investigations, together with those previously noted, suggest that CP directly stimulates marrow stem cells and/or CFC to self-replicate, thus increasing the host

Table 3

Effect of CP with CY on macrophage colony production by BMC's from tumor-bearing mice

Dose of CY[a]	Days of treatment	No. of experiments	BMC's/femur (× 10[6]) 0.9% NaCl solution	BMC's/femur (× 10[6]) CY + CP	No. of colonies/plate (1 × 10[5] BMC's) 0.9% NaCl solution (A)	No. of colonies/plate (1 × 10[5] BMC's) CY + CP (B)	No. of colonies/plate (1 × 10[5] BMC's) % (B/A)	No. of colonies/femur (× 10[3]) 0.9% NaCl solution (C)	No. of colonies/femur (× 10[3]) CY + CP (D)	No. of colonies/femur (× 10[3]) %(D/C)
1	0									
	4[b]									
2	7	4	10.8[c] ± 0.16	8.3 ± 0.47	65.6 ± 5.82[d]	108.2 ± 5.44	165[e]	6.9 ± 0.59	8.9 ± 0.58	128[f]
	11	6	9.1 ± 0.64	3.8 ± 0.50	71.9 ± 5.48	214.9 ± 25.76	298[e]	6.3 ± 0.33	7.5 ± 0.70	118
3	14	3	10.9 ± 0.66	9.6 ± 0.86	109.0 ± 3.73	149.2 ± 12.94	136[e]	11.6 ± 0.40	13.8 ± 0.84	119[f]
	18	5	11.0 ± 0.96	4.5 ± 1.35	45.6 ± 4.12	175.4 ± 24.49	384[e]	5.1 ± 0.37	5.2 ± 0.24	102
4	21	3	12.2 ± 0.81	8.9 ± 2.32	36.3 ± 1.57	77.7 ± 1.93	214[e]	4.5 ± 0.25	7.0 ± 0.57	155[e]
	25	2	11.3	4.6	53.6 ± 2.38	140.1 ± 10.72	261[e]	6.1 ± 0.55	6.5 ± 0.30	106
7	42	2	14.9	12.3	21.1 ± 2.67	42.0 ± 2.56	199[e]	3.1 ± 0.39	4.3 ± 0.26	138[f]
	46	2	11.9	3.8	45.5 ± 4.62	217.9 ± 12.32	478[e]	6.7 ± 0.68	15.9 ± 0.90	236[e]
8	49	2	14.1	12.3	49.3 ± 4.99	84.2 ± 4.22	170[e]	6.5 ± 0.47	10.0 ± 0.68	153[e]
	53	3	11.0 ± 0.83	7.3 ± 1.62	54.8 ± 2.83	168.4 ± 20.43	307[e]	6.2 ± 0.43	12.8 ± 1.68	204[f]

[a] CY, 90 mg/kg, every 7 days; and CP, 1.4 mg, every 7 days.
[b] First dose CP.
[c] Mean ± S.E.
[d] Mean ± S.E. of total plates; 10/experiment.
[e] $p < 0.001$.
[f] $p < 0.005$.

capability for macrophage production, but does not selectively influence the cytotoxicity of those cells. Since the proportion of cytotoxic cells remains constant, it would seem that CP exerts its effect on bone marrow cells in a nonspecific manner, in contrast to its specific effect on lymph node cell cytotoxicity (7).

Although the proportion of cytotoxic cells was not altered by the CP being administered with CY, the absolute number of such cells was increased by virtue of the proliferation of CFC. Since the increase in macrophage colony production (and, consequently, in cytotoxic macrophages) obtained when CP was used with CY correlates with increased tumor growth inhibition, it is suggested that macrophage precursors are the cells of primacy in CP immunopotentiation. Their stimulation, resulting in enhanced cytotoxic macrophage formation, could be responsible for the augmented inhibition of tumor growth observed in our model system.

A number of other considerations have resulted as a consequence of these investigations. It had previously been postulated by us that the transient duration of the enhanced CFC production in tumor-bearing mice was the result of a negative feedback inhibition and that the increase in CFC occurring in mice treated with CP at a time when stimulation due to the tumor had already ceased was, at least in part, the result of the interruption of such a mechanism by the CP. The present finding indicating that, when CP was administered, CFC production was increased over that which resulted in response to the deficit induced by CY suggests that the effect of CP under such circumstances is probably the result of direct stimulation of CFC or their precursors by the CP, because it is unlikely that a negative feedback mechanism is operational at a time when the hematopoietic insult by CY had not yet been corrected.

Direction of attention only toward an assessment of numbers of marrow cells present following CY therapy with or without CP would have resulted in the erroneous conclusion that CP was without beneficial effect on bone marrow, since there was no evidence that numbers of bone marrow cells had been increased or prevented from decreasing by the administration of CP. In fact, the number of bone marrow cells per femur was slightly less when CP was administered with CY than when CY alone was used. The latter observation is of particular interest in view of recent findings by us (9) indicating that, when CP was used with CY, an alteration in the metabolism of CY resulted. With ^{14}C-labeled CY, it was found that, while total plasma radioactivity was similar in mice treated either with CY alone or with CP and CY, the proportion of total activity due to nonmetabolized CY was greater when CP was used. The possibility is considered that, because of the slowing of metabolism following CP, a more sustained CY effect results which could be associated with a more profound myelosuppression. Perhaps this might at least in part account for the observed slight decrease in number of bone marrow cells when CP was given with CY. Whatever the explanation, the findings may seem antithetical to the premise that CP may attenuate myelosuppression due to chemotherapy (14), since they indicate that CP stimulates the number of cells entering into differentiation while only slightly affecting the total bone marrow population. The lack of an increase in, or even prevention of a loss of, BMC's during the time period of

Table 4

Effect of CY with and without CP on macrophage colony production by BMC's from tumor-bearing mice

Dose of CY[a]	Days of treatment	No. of experiments	BMC's/femur (× 10^6)		No. of colonies/plate (1 × 10^5 BMC's)			No. of colonies/femur (× 10^3)		
			CY	CY + CP	CY (A)	CY + CP (B)	% (A/B)	CY (C)	CY + CP (D)	% (C/D)
1	0									
	4[b]									
2	7	3	9.1[c] ± 0.12	8.0 ± 0.52	65.0 ± 9.1[d]	95.4 ± 4.11	146[e]	6.2 ± 0.53	7.4 ± 0.36	118[f]
	11	3	6.3 ± 0.72	4.2 ± 0.63	94.1 ± 6.12	137.3 ± 8.55	145[e]	3.6 ± 0.17	5.5 ± 0.20	150[e]
3	14	3	13.3 ± 0.80	9.6 ± 0.86	65.2 ± 6.2	150.9 ± 12.35	231[e]	8.7 ± 0.80	13.8 ± 0.84	159[e]
	18	3	4.8 ± 2.16	6.3 ± 1.42	68.8 ± 3.71	81.9 ± 3.71	119[f]	3.0 ± 0.20	4.7 ± 0.30	153[e]
4	21	3	10.1 ± 2.54	8.8 ± 2.32	63.1 ± 1.57	77.7 ± 1.93	123[e]	6.5 ± 0.53	7.0 ± 0.57	107
	25	2	5.5	4.5	111.1 ± 8.84	140.1 ± 10.72	126[f]	5.2 ± 0.29	6.5 ± 0.30	125[f]
5	28	2	12.4	12.8	68.2 ± 6.38	141.0 ± 8.82	206[e]	8.0 ± 1.33	17.7 ± 1.11	220[e]
	32	2	4.4	8.2	153.8 ± 11.06	185.2 ± 18.84	120	6.8 ± 0.49	16.4 ± 1.67	242[e]
7	42	2	12.4	12.3	34.7 ± 5.55	49.7 ± 5.29	143	4.8 ± 0.66	6.1 ± 0.84	127
	46	2	3.8	3.8	89.0 ± 9.17	267.9 ± 42.22	301[e]	4.7 ± 0.58	16.2 ± 0.58	339[e]
8	49	2	12.4	12.3	51.1 ± 2.73	84.2 ± 4.22	164[e]	6.3 ± 0.36	10.0 ± 0.68	158[e]
	53	3	7.0 ± 0.90	7.3 ± 1.61	86.3 ± 5.77	168.4 ± 20.43	195[e]	7.7 ± 0.97	12.8 ± 1.68	165[f]
9	56	4	14.2 ± 0.48	14.6 ± 0.62	46.9 ± 2.03	91.9 ± 4.17	195[e]	6.5 ± 0.30	13.5 ± 0.70	206[e]
	60	2	3.2	3.7	44.7 ± 2.76	122.0 ± 9.13	272[e]	1.4 ± 0.08	4.4 ± 0.33	312[e]
10	63									
	67	2	5.1	5.4	133.7 ± 13.75	245.3 ± 15.73	183[e]	6.7 ± 0.69	13.3 ± 0.85	196[e]
12	77	2	18.5	14.5	28.7 ± 2.91	58.7 ± 3.89	205[e]	5.4 ± 0.60	8.4 ± 0.47	155[e]
	81	2	5.0	3.7	75.2 ± 9.10	148.1 ± 10.73	196[e]	3.7 ± 0.45	5.4 ± 0.39	144[e]

[a] CY, 90 mg/kg, every 7 days; and CP, 1.4 mg, every 7 days.
[b] First dose CP.
[c] Mean ± S.E.
[d] Mean ± S.E. of total plates; 10/experiment.
[e] $p < 0.001$.
[f] $p < 0.005$.

these observations is probably indicative of the rapid egress of CFC and their progeny from marrow in response to the need for elimination of extramedullary deficits. While the present studies have not been carried out to determine whether there is a more rapid restoration of the total marrow cell population to normal with the use of CP, it might be anticipated that such occurs. Thus, it would seem that when myelosuppression is a limiting factor in the use of a chemotherapeutic agent, the concomitant use of CP may be advantageous. However, the use of total cellularity as an index of the CP marrow-sparing effect may be misleading.

REFERENCES

1. Basic, I., Milas, L., Grdina, D. J., and Withers, I. R. Destruction of Hamster Ovarian Cell Cultures by Peritoneal Macrophages from Mice Treated with *Corynebacterium granulosum*. J. Natl. Cancer Inst., *52:* 1839–1842, 1974.
2. Baum, M., and Fisher, B. Macrophage Production by the Bone Marrow of Tumor-bearing Mice. Cancer Res., *32:* 2813–2817, 1972.
3. Bradley, T. T., and Metcalf, D. The Growth of Mouse Bone Marrow Cells *in vitro*. Australian J. Exptl. Biol. Med. Sci., *44:* 287–299, 1966.
4. DeWys, W. D., Goldin, A., and Mantel, N. Hematopoietic Recovery after Large Doses of Cyclophosphamide: Correlation of Proliferative State with Sensitivity. Cancer Res., *30:* 1692–1697, 1970.
5. Eccles, S. A., and Alexander, P. Macrophage Content of Tumours in Relation to Metastatic Spread and Host Immune Reaction. Nature, *250:* 667–669, 1974.
6. Evans, R. Macrophages in Syngeneic Animal Tumours. Transplantation, *14:* 468–473, 1972.
7. Fisher, B., Wolmark, N., and Coyle, J. Effect of *Corynebacterium parvum* on Cytotoxicity of Regional and Non-regional Lymph Node Cells from Animals with Tumors Present or Removed. J. Natl. Cancer Inst., *53:* 1793–1801, 1974.
8. Fisher, B., Wolmark, N., Coyle, J., and Saffer, E. The Effect of a Growing Tumor and Its Removal on the Cytotoxicity of Macrophages from Cultured Bone Marrow Cells. Cancer Res., *36:* 2302–2305, 1976.
9. Fisher, B., Wolmark, N., Rubin, H., Levine, M., and Saffer, E. Further Observations on the Inhibition of Tumor Growth by C. parvum with Cyclophosphamide: III. Effect of C. parvum on Cyclophosphamide Metabolism. J. Natl. Cancer Inst., in press.
10. Fisher, B., Wolmark, N., Rubin, H., and Saffer, E. Further Observations on the Inhibition of Tumor Growth by *Corynebacterium parvum* with Cyclophosphamide: I. Variation in Administration of Both Agents. J. Natl. Cancer Inst., *55:* 1147–1153, 1975.
11. Fisher, B., Wolmark, N., Saffer, E., and Fisher, E. R. Inhibitory Effect of Prolonged *Corynebacterium parvum* and Cyclophosphamide Administration on the Growth of Established Tumors. Cancer, *35:* 134–143, 1975.
12. Fried, W. and Johnson, C. The Effect of Cyclophosphamide on Hematopoietic Stem Cells. Radiation Res., *36:* 521–527, 1968.
13. Ghaffar, A., Cullen, R. T., Dunbar, N., and Woodruff, M. F. A. Antitumour Effect *in vitro* of Lymphocytes and Macrophages from Mice Treated with *Corynebacterium parvum*. Brit. J. Cancer, *29:* 199–205, 1974.
14. Israel, L., and Edelstein, R. Non-specific Immunostimulation with *Corynebacterium parvum* in Human Cancer. *In:* Immunological Aspects of Neoplasia, The University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, pp. 485–504. Baltimore: The Williams & Wilkins Co., 1975.
15. Kaul, M., and Hudson, G. Cell-degeneration Pattern in Bone Marrow and Blood of Guinea Pigs following Administration of Cyclophosphamide. Acta Haematol., *42:* 42–49, 1969.
16. Keller, R., and Hess, M. W. Tumour Growth and Non-specific Immunity in Rats: The Mechanisms Involved in Inhibition of Tumour Growth. Brit. J. Exptl. Pathol., *53:* 570–577, 1972.
17. Olivotto, M., and Bomford, R. *In vitro* Inhibition of Tumour Cell Growth and DNA Snythesis by Peritoneal and Lung Macrophages from Mice Injected with *Corynebacterium parvum*. Intern. J. Cancer, *13:* 478–488, 1974.
18. Tevethia, S. S., and Zarling, J. M. Participation of Macrophages in Tumor Immunity. Natl. Cancer Inst. Monograph, *35:*279–282, 1972.
19. Valeriote, F. A., Collins, D. C., and Bruce, W. R. Hematological Recovery in the Mouse following Single Doses of Gamma Radiation and Cyclophosphamide. Radiation Res., *33:* 501–511, 1968.
20. Wolmark, N., and Fisher, B. The Effect of a Single and Repeated Administration of *Corynebacterium parvum* on Bone Marrow Macrophage Colony Production in Syngeneic Tumor Bearing Mice. Cancer Res., *34:* 2869–2872, 1974.
21. Wolmark, N., Levine, M., and Fisher, B. Effect of a Single and Repeated Administration of *Corynebacterium parvum* on Bone Marrow Macrophage Colony Production in Normal Mice. Res. J. Reticuloendothelial Soc., *16:* 252–257, 1974.

Correlation of Antitumor Chemoimmunotherapy with Bone Marrow Macrophage Precursor Cell Stimulation and Macrophage Cytotoxicity

Bernard Fisher and Norman Wolmark

Cancer Res 1976;36:2241-2247.