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Responsiveness of Senescent Mice to the Antitumor Properties of *Corynebacterium parvum*[1]

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SUMMARY

The antitumor properties of *Corynebacterium parvum* have been studied in young (3- to 8-month-old) and aged (18 or more months old) BALB/c mice given s.c., i.m., i.p., or i.v. transplants of the highly malignant, weakly immunogenic line 1 lung carcinoma, and in aged (25- to 33-month-old) BALB/c mice bearing primary mammary tumors. These aged BALB/c mice were shown to be less immunoresponsive than their younger counterparts, and this, in combination with nonimmunological factors, made them more sensitive to the lethal effects of the line 1 carcinoma. Correspondingly, *C. parvum* proved to have less antitumor activity in aged mice than it did in young mice. In spite of this relatively weaker antitumor activity for *C. parvum* in aged mice, repeated injections of this agent were able to induce temporary regressions of the primary mammary tumors studied and thereby prolong survival time.

INTRODUCTION

Most experimental studies on specific and nonspecific immunotherapy are conducted in young adult mice bearing strongly immunogenic transplanted tumors. In this respect, they bear little similarity to the clinical situation in that the primary tumors encountered in the clinic are seldom strongly immunogenic, and host immune responsiveness is reduced due to age, tumor growth, side effects of therapy, or their combination. That these differences might be of major importance was suggested by the data of Milas *et al.* (2, 3), in which it was shown that weakly immunogenic tumors were less responsive to the antitumor properties of both *Corynebacterium granulosum* and CP[3] and that total-body irradiation interfered with both the prophylactic and therapeutic action of these immunostimulants. The present report extends these observations and compares, in young and old mice, the ability of CP to inhibit (*a*) the transplanted weakly immunogenic line 1 lung carcinoma and (*b*) primary mammary tumors growing in aged BALB/c mice.

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[3] The abbreviations used are: CP, *Corynebacterium parvum;* PBS, phosphate-buffered saline (Dulbecco's: 100 mg $CaCl_2$ + 200 mg KCl + 200 mg KH_2PO_4 + 100 mg $MgCl_2 \cdot 6H_2O$ + 8 g NaCl + 1.15 g $Na_2HPO_4 \cdot 2H_2O$ per liter); PEC, peritoneal exudate cell.

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MATERIALS AND METHODS

Mice. The mice used in this study were specific-pathogen-free BALB/c males and females. A single sex was used within an experiment, and we have yet to detect a sex difference in terms of responsiveness to either CP or the line 1 carcinoma. Recipients of the line 1 carcinoma ranged in age from 3 to 23 months. For certain of the experiments, female BALB/c mice bearing primary mammary tumors were used. These mice had been exposed to graded doses and dose rates of ^{137}Cs γ-rays at the age of 4 months (8) and were 26 to 33 months old at the time of CP treatment. All of these mice were introduced into the experiment at the same time and were randomly assigned to receive either PBS or CP. Mice were housed 8 or less per cage, with food and chlorinated water provided *ad libitum.*

CP. Killed vaccines of this bacteria (Burroughs-Wellcome Inc., Research Triangle Park, N. C.; CN-6134, Batch PX-374) were kindly provided by Dr. John K. Whisnant. The suspension (7 mg/ml, dry weight) was diluted with PBS and, unless otherwise stated, a single i.p. injection of 0.25 mg in 0.5 ml was given.

Line 1 Lung Carcinoma. The origin and maintenance of this highly malignant, weakly immunogenic cancer of the type II alveolar cell of BALB/c mouse lung have been given elsewhere (10). All tumor cell suspensions were prepared from 10-day-old s.c. transplants, with the exception of those cells used to study tumor cell lodging and tumor clone development in the lungs. These were obtained from 3-day-old *in vitro* cultures as described elsewhere (9).

Tumor Growth and Metastasis Assays. The growth of s.c. transplants of the line 1 carcinoma was assayed by surgically removing tumor specimens and weighing them. Artificial metastases, or the development of lung tumor colonies from i.v.-injected cells, were assayed by killing the mice 21 days after injection and clearing and staining the lungs (7). Spontaneous metastases were scored 35 days after s.c. transplant by the same technique for the lungs, and by gross and microscopic examination of all other tissues.

The growth of primary mammary tumors was determined by measuring the 3 diameters of the tumor, calculating the tumor volume, and expressing it as a multiple of the tumor volume at the start of the experiment. Mean tumor volume differed by less than 8% between the PBS- and CP-treated groups at the start of the experiment.

Tumor Cell Labeling and Assay. Line 1 carcinoma cells, obtained from 3-day *in vitro* cultures, were labeled with ^{51}Cr

according to standard techniques. Labeled cells (2×10^4) were injected i.v. into recipients of various ages, and 24 hr later the lungs were harvested and the contained radioactivity was determined. In agreement with the data of Withers and Milas (6), but in contrast to the data of Brown (1), we found that ^{51}Cr-labeled cells provided an accurate estimate of the tumor cells that had lodged in the lungs; *i.e.*, injection of free ^{51}Cr label resulted in less than 1% of the activity observed in the lungs of mice given similar amounts of the label attached to tumor cells.

PEC Harvest. PEC's were harvested from mice by means of peritoneal lavage, with 3 ml of Eagle's basal medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (i.p. at 4°), and then the fluid was collected. CP does not induce ascites in our BALB/c mouse as it does in certain other mouse strains (3). Routinely, 70 to 80% of these PEC's adhere to glass and are morphologically indistinguishable from macrophages.

RESULTS

PEC Concentrations. Chart 1 is a plot of the number of PEC's in young and old mice, both prior to and for the 1st 14 days after CP injection. Three points are readily apparent from the data: (*a*) young (3-month-old) mice possess a greater number of PEC's than do aged (22-month-old) mice, even in the absence of CP stimulation; (*b*) the time course of the increase and decline of PEC's following CP treatment is the same in young and old mice; and (*c*) at all time intervals after CP treatment, the absolute number of PEC's is higher in the younger group, but both groups show the same response to CP in terms of percentage increases relative to the unstimulated level. Therefore, aged mice possess fewer PEC's than do young mice, but their response to CP is similar on a percentage basis.

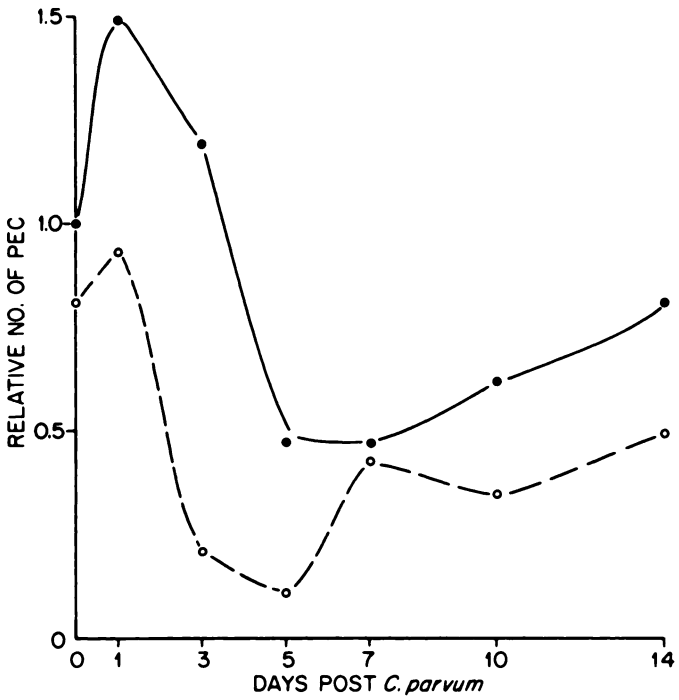

Chart 1. Number of PEC's obtained from untreated mice and from mice given 0.25 mg of CP 1 to 14 days earlier. ●, 3-month-old BALB/c mice; ○, 22-month-old BALB/c mice. (○).

CP Tumor Inhibition *versus* Transplant Route and Age. Table 1 contains the results of an experiment in which 3-, 8-, or 23-month-old mice were given either PBS or 0.25 mg of CP, and 7 days later were challenged with 10^5 line 1 carcinoma cells by the s.c., i.m., i.p., or i.v. route. The data for the 3- and 8-month-old mice were not significantly different in any of the comparisons, and the pooled estimate, referred to as the 3- to 8-month group, was used. Furthermore, within an age and treatment group, the data for s.c. and i.m. challege routes were identical; again the data were pooled, yielding the s.c.-i.m. challenge group (Table 1).

All of the mice given PBS prior to tumor challenge died, the mean survival time being a function of both recipient age and challenge route (Table 1). The i.v.-injected cells killed the mice more rapidly than those given i.p., and the longest survival times were observed in the s.c.-i.m. groups. For each of the challenge routes, the PBS-treated aged mice died 10 to 14 days earlier than their younger counterparts.

In neither age group did CP treatment have a significant effect on the mortality or survival time of mice given the line 1 carcinoma by the s.c.-i.m. route but, at both ages, injection of CP significantly reduced mortality or increased survival time or both when the tumor was given either i.p. or i.v. (Table 1). Overall, the younger mice appeared more responsive to CP than did the aged mice. Although CP significantly increased survival time in aged mice given i.p. tumor, it did not allow any of these mice to survive through the termination of the experiment. In contrast, CP reduced the mortality in the i.p.-challenged young mice from 100% to essentially zero. A similar age dependence was obseved for the ability of CP to alter the response to i.v. injections of the line 1 carcinoma (Table 1). Further studies were conducted in which the challenge tumor cell dose was both increased and decreased, and in which the time between CP and tumor challenge was altered. All of these data were consistent with the conclusions reached from the data in Table 1, *i.e.* (*a*) aged mice are more sensitive to the lethal effects of the line 1 carcinoma, (*b*) aged mice are less responsive to the tumor-inhibitory properties of CP, and (*c*) CP, when given 7 days before tumor challenge, does not affect the survival patterns of mice given an s.c. or i.m. tumor challenge.

Tumor-Host-CP Interactions. Although s.c. transplants of the line 1 carcinoma grow slower in aged mice than in young adults (10), the aged mice die sooner (Table 1). Resolution of this paradox can be seen in Table 2, which contains the results of a spontaneous metastasis assay conducted in 3-, 9-, and 18-month-old mice bearing s.c. transplants of the tumor. Spontaneous metastasis to the lungs and elsewhere proceeds more rapidly in the aged mice than it does in either of the younger groups (Table 2). These data support our earlier conclusion (9) that acceleration of spontaneous metastases results in a reduced growth rate for the s.c. implant and in a shortened survival time.

Table 1
Effects of CP on the survival of BALB/c mice given 10^5 line 1 carcinoma cells by various routes

Transplant route	Age (mo.)	Controls		CP-treated[a]	
		Deaths[b]	MST[c] (days)	Deaths[b]	MST[c] (days)
s.c.-i.m.[d]	3–8	31/31	74.0 ± 3.0	32/32	82.7 ± 3.5
i.p.	3–8	15/15	61.0 ± 2.8	1/14	136
i.v.	3–8	15/15	54.1 ± 6.1	9/23	102.2 ± 8.4
s.c.-i.m.	23	16/16	64.0 ± 5.1	13/13	66.5 ± 2.8
i.p.	23	7/7	51.4 ± 8.0	6/6	90.2 ± 13.8
i.v.	23	8/8	40.5 ± 4.6	4/8	56.3 ± 12.4

[a] Injection of 0.25 mg of CP (i.p.) 7 days before transplant.
[b] Number of deaths/number of mice given injections.
[c] MST, mean survival time of decedents ± S.E.
[d] Pooled results of s.c. and i.m. injection-treated mice.

Table 2
Frequency of spontaneous metastases from s.c. transplants of the line 1 carcinoma

Recipient age (mo.)	Time after transplant (days)	Frequency[a] of metastases in	
		Lungs	Other organs
3	21	0/8	0/8
9	21	0/8	0/8
18	21	3/4	0/4
3	28	2/7	0/7
9	28	2/7	0/7
18	28	4/6	2/6
3	35	8/8	0/8
9	35	6/7	1/7
18	35	5/5	5/5

[a] Number of mice with a metastasis/number of mice examined.

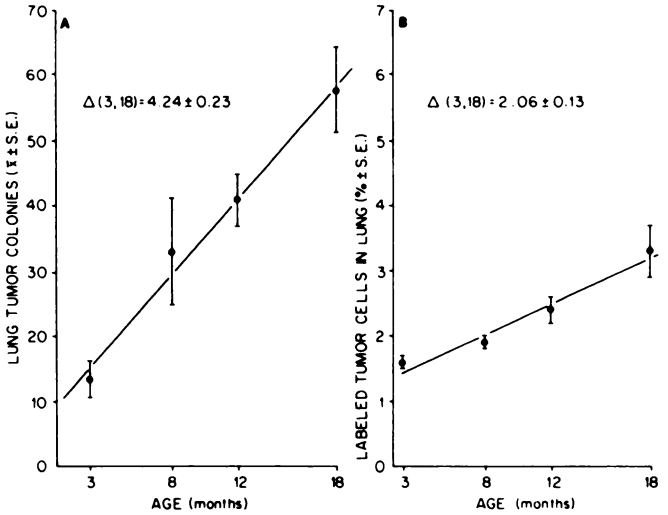

Chart 2. *A*, mean number of lung tumor colonies 21 days after the injection of 2 × 10^4 line 1 carcinoma cells as a function of recipient age; *B*, mean radioactivity in the lungs 24 hr after the injection of 2 × 10^4 ^{51}Cr-labeled line 1 carcinoma cells as a function of recipient age.

Chart 2*A* is a plot of the number of lung tumor colonies observed 21 days after i.v. injection of 2 × 10^4 line 1 tumor cells in mice of varying ages. As the age of the recipient increases, the number of colonies that develop increases linearly over the age range of 3 to 18 months (Chart 2*A*). Between these 2 extremes, the number of colonies that develop, following the injection of the same number of tumor cells, increases by a factor of 4.2. In Chart 2*B* is a plot of the radioactivity detected in the lungs 24 hr after the injection of ^{51}Cr-labeled tumor cells, with recipient age again ranging from 3 to 18 months. A linear increase in lodged tumor cells with increasing recipient age was observed, with the factor increase between 3 and 18 months of age being 2.1 (Chart 2*B*). When the i.v. tumor cell dose given to aged (18-month-old) mice is reduced, so as to yield lower numbers of tumor colonies, survival times are the same as those for young mice with a similar lung tumor colony burden, but responsiveness to CP remains less than that of their younger counterparts.

While the data in Chart 1 would suggest that the time course of response to CP is the same in young and old mice, these data are for a single cell type which may or may not be the prime determinant of CP's antitumor activity. Chart 3 is a plot of the survival time of young (3-month-old) and aged (22-month-old) mice challenged with 10^6 line 1 carcinoma cells at varying times after the standard injection of CP. These curves are essentially identical to those obtained for PEC development, but are displaced 2 days later.

The failure of CP, when given 7 days prior to s.c.-i.m. transplant, to affect survival of both young and aged hosts suggests that this treatment does not affect the rate of development of spontaneous metastases, which are the proximate cause of death in this system (9). This point was confirmed in 5 separate experiments (Table 3). In these mice, which served as controls in other experiments, CP injection 7 days prior to s.c. tumor transplant failed to affect either the size of the s.c. tumor on Day 21 or the frequency of spontaneous metastasis determined on Day 35. Correspondingly, the survival time of parallel groups was not increased ($p < 0.80$).

CP Treatment of Primary Mammary Tumors. Table 4 summarizes the characteristics of and survival data for each

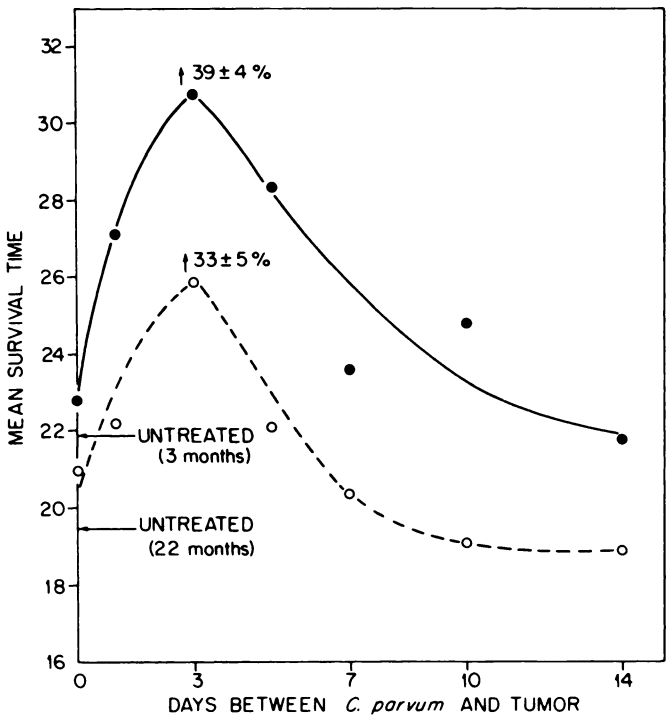

Chart 3. Mean survival times of untreated 3- (●) and 22- (○) month-old BALB/c mice given 10^6 line 1 carcinoma cells i.p., and of similar mice given 0.25 mg of CP 0 to 14 days earlier.

of the 17 mice used in the primary summary tumor therapy experiment. Although each of the mice had been exposed to γ-rays as young adults (8), we hesitate to refer to these tumors as being radiation induced due to the negligible increase in incidence associated with irradiation. On Day 0 of the experiment 9 mice were given PBS injections (0.50

Table 3

Effects of CP on the growth and spontaneous metastasis of s.c.-transplanted line 1 carcinoma cells

Treatment	21-day tumor wt (mg)	35-day spontaneous lung metastases
None	1642 ± 215[a]	17.0 ± 3.5
CP	1526 ± 224	12.8 ± 3.0
None	2073 ± 204	32.5 ± 3.1
CP	2160 ± 392	25.0 ± 4.8
None	2671 ± 132	ND[b]
CP	2326 ± 163	ND
None	3453 ± 480	8.3 ± 1.2
CP	3553 ± 359	13.0 ± 3.0
None	3674 ± 476	ND
CP	3254 ± 340	ND

[a] Mean ± S.E.
[b] ND, not determined.

Table 4

Response to CP immunotherapy of BALB/c mice bearing autochthonous mammary tumors

Age at start of experiment (mo.)	Radiation exposure at age 4 mo.		Treatment[a]	Survival time[b] (days)	Metastases from mammary tumors	Other tumors[c]
	Dose (rads)	Dose rate (rads/day)				
32	392	Acute	PBS	10	None	RCS, T-ova
28	49	Acute	PBS	21	None	RCS
33	98	112	PBS	31	None	T-ova, T-lung, T-adr
26	98	14	PBS	44	None	None
30	196	1.75	PBS	44	None	RCS, T-lung
28	196	7	PBS	51	None	T-ova, T-hdr
28	196	3.5	PBS	59	Lung	RCS
27	49	7	PBS	70	None	None
26	49	14	PBS	87	None	T-lung, T-fbs
32	49	Acute	CP	17	None	None
27	49	7	CP	30	None	None
30	196	1.75	CP	34	None	T-adr
27	98	3.5	CP	76	None	RCS
27	49	3.5	CP	80	None	RCS, T-lung, T-adr
33	98	112	CP	92	None	T-ova
27	196	14	CP	106	Lung	RCS
27	392	Acute	CP	114	None	None

[a] PBS or 0.25 mg of CP given on Days 0, 7, and 21.
[b] Calculated from the start of therapy.
[c] T-hdr, harderian gland tumor; T-adr, adrenal tumor; and T-fbs, fibrosarcoma; T-ova, tumor of the ovary; RCS, reticulum cell sarcoma.

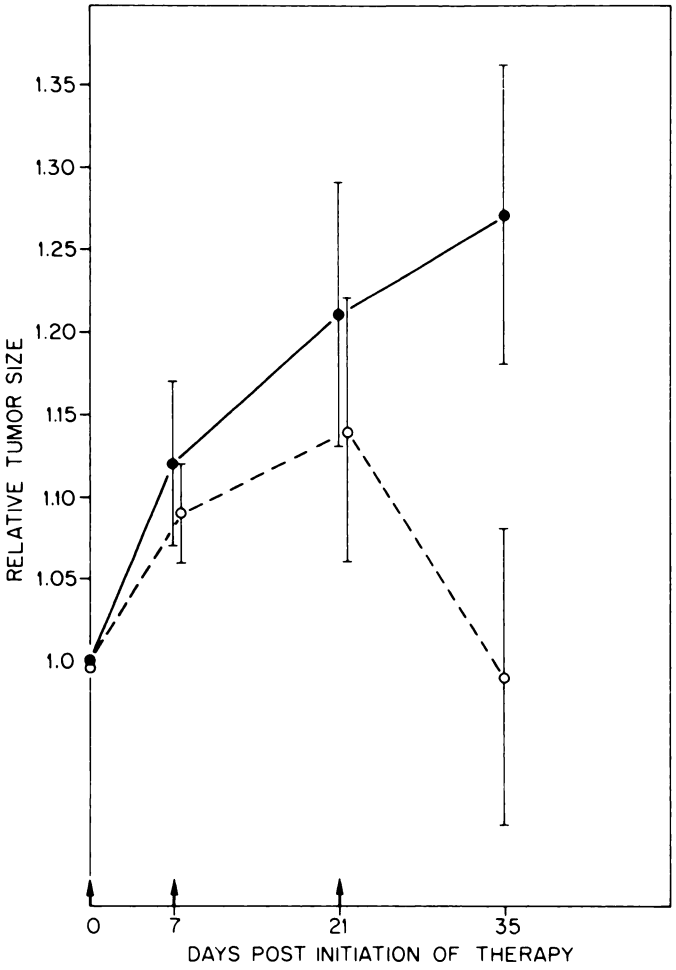

Chart 4. Mean size of mammary tumors as a function of time after start of therapy. Controls (●) received 0.5 cu cm of 0.9% NaCl solution on Days 0, 7, and 21, while CP-treated mice (○) received i.p. injections of 0.25 mg of CP on the same days.

ml) and 8 were given 0.25 mg of CP in the same vehicle. The treatments were repeated on Day 7 and again on Day 21 of the experiment. Chart 4 is a plot of the relative tumor sizes during the 1st 35 days of the experiment. The 1st and 2nd CP injections slowed the growth of the tumors, but only after the 3rd injection had been given was a true regression observed (Chart 4). Although the data in Chart 4 are for all of the mice within each group, similar growth patterns would be observed if we deleted those that died early, *i.e.*, the onset of regressions in the CP group is not due to population selection. If we consider only those mice that survived through the time that regression was observed in the CP group (Day 40), a statistically significant increase in survival times was observed ($p < 0.02$).

At the times of death, tumors were approximately the same size in the 2 groups, and metastasis was rare in both. This would suggest that CP promoted regression of the tumors, but that, following termination of treatment, the tumors returned to an actively growing state.

DISCUSSION

For this discussion, we define an aged BALB/c mouse as one that is at least 18 months old. We have shown elsewhere (10) that both humoral and cellular immune competence in these aged mice is only 10 to 20% of that observed in young adults. Furthermore, aged BALB/c mice show the same age-dependent return of sensitivity to oncogenic viruses in old age that we have previously demonstrated for the BALB/c × DBA/2 F_1 mouse (4). This return of sensitivity in old age results from the decline of multiple facets of immune competence, including both T- and B-lymphocyte function. These data are presently being expanded and will be reported elsewhere.

Although CP is an acknowledged immunostimulant (5) and aged mice are less immunoresponsive than young adults, it does not follow that the greater sensitivity of aged mice to the line 1 carcinoma and the poorer response of these mice to the antitumor properties of CP are strictly immunological phenomena. As an example, the lodging of tumor cells within the lungs within the 1st 24 hr after injection (Chart 2*B*) increases with advancing age, thereby resulting in a larger developing tumor burden in the aged animals. We consider it unlikely that immunological phenomena are responsible for this difference, which occurs within the 1st 24 hr, and consider it more likely that a physical factor within the lung increases the efficiency of trapping (6). This increased tumor burden in aged mice, in itself, could decrease the antitumor properties of CP, since the effectiveness of this type of immunotherapy is inversely related to the tumor burden (5). This cannot be the total explanation, however, since aged mice are less responsive to CP even when their tumor burden is adjusted to equal that of young adults.

In the line 1 carcinoma system, injection of CP 7 days prior to s.c. tumor transplant fails to affect survival (Table 1), s.c. tumor size, or the development of spontaneous metastases (Table 3). Since the effectiveness of CP decays rapidly in this system (Charts 1 and 3) as opposed to more prolonged antitumor activity observed by others (2, 3, 5), we propose that CP fails to affect the growth of these s.c. tumors because the net tumor antigen burden (strength × quantity) is insufficient to exploit the adjuvant properties of CP during the early, stimulated phase, and that it fails to affect spontaneous metastasis because the stimulation has waned prior to their development (11).

In spite of these limitations, it is apparent that CP, under appropriate conditions, can induce temporary regression of primary mammary tumors growing in old, previously irradiated hosts, and thereby prolong their survival (Chart 4; Table 4). It would appear, therefore, that CP remains a potential means of treating clinical cancer, inasmuch as we have observed antitumor activity with it under conditions that approach those of the clinic.

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