Europ. J. Cancer Vol. 15, pp. 867–873

0014-2964/79/0601-0867 $02.00/0

Surgery Plus *Corynebacterium parvum* Immunotherapy for Lewis Lung Carcinoma in Mice

K. KARRER,* W. RELLA* and A. GOLDIN†

Institute for Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Wien, Austria
†*The National Cancer Institute, Bethesda, Maryland, U.S.A.*

Abstract—*The effect of the immunoadjuvant* Corynebacterium parvum (C. parvum) *on residual spontaneous micrometastases was studied in C57Bl/6 × DBA/2 hybrid mice carrying an intramuscular inoculum of syngeneic Lewis lung carcinoma cells. The "primary" tumor was allowed to grow for* 3, 5, 7, 10 *or* 12 days *and was then removed by surgery.* C. parvum *was administered either i.p. or s.c. (proximal to the local tumor) using a dose of* 175–525 μg *per mouse. The animals were treated* 3 days *before surgery, on the day of surgery or* 5 days *thereafter.*

The effect of treatment was best seen when surgery was performed as early as 5 days *after tumor inoculation. Deaths from metastases were prevented, at this time, by surgery alone in* 45% *of the control animals whereas up to* 90% *of adjuvantly treated animals were cured. With increasing load of "primary" tumor and metastases in the mice, the effectiveness of* C. parvum *treatment was overwhelmed and no further reduction of tumor growth was seen.*

C. parvum *was more effective when given* 3 days *before amputation, than when given* 5 days *after amputation. The i.p. route of* C. parvum *was not as effective as local treatment at the area of amputation. The relative rate of local tumor-recurrences was not reduced by the treatment and was, in fact, frequently increased.*

These results demonstrate a definite, but rather limited range of effectiveness of C. parvum *in this tumor model system depending on the tumor burden as well as on the timing of treatment.*

INTRODUCTION

Corynebacterium parvum is recognized to be an important immunoadjuvant [1, 2]. Suspensions of killed *C. parvum* have been shown to have a marked stimulatory effect on the lymphoreticular system [3–5], This agent, non-pathogenic by itself, can activate the immune system with resultant destruction of cells with abnormal growth characteristics [6–8]. Benefit from the treatment with *C. parvum* have been described in a number of animal tumor model systems [9–12]. However, little is known about the relevance to the human situation [13–15]. The predominant problem in the treatment of cancer of man is the prevention of metastases after successful removal of the primary tumor. The experimental design employed in this study is aimed at simulating the clinical situation as closely as possible: an intramuscularly inoculated "primary" tumor was allowed to grow in the syngeneic host for varying periods of time; then the local tumor was surgically removed, and the effect of *C. parvum* was determined on the growth of residual metastatic cells.

Accepted 22 November 1978.
Key words: Lewis lung carcinoma—surgery—adjuvant immunotherapy—*Corynebacterium parvum*.
This study was supported by the "Fonds fur Förderung der wissenschafflichen Forschung", Austria.

MATERIALS AND METHODS

Animals

Male hybrid mice (C57Bl/6 × DBA/2), weighing 20–25 g, were obtained from the Charles River Breeding Colony at Calco, Italy. The animals were kept in plastic cages and fed commercial food pellets and water *ad libitum*.

Tumor

Lewis lung carcinoma, a syngeneic transplantable tumor which originated spontaneously in C57Bl/6 mice, served as the tumor model. Lung metastases develop in all animals soon after tumor inoculation and spontaneous regressions have never been observed. By surgical removal of the "primary" tumor at different times after tumor inoculation, animals were left with different numbers of spontaneous micrometastases, thus resembling the clinical condition of minimal residual disease. The suitability of this model system for cancer treatment experiments has been previously documented [16–19].

Tumor inocula were prepared from large, non-ulcerated 8–10-day old subcutaneous tumors by forcing fragments of the tumor through a 20 gauge needle. Twenty millitres of this tumor mash were suspended in 80 ml of 0.9% NaCl containing 500 units/ml of penicillin and 250 μg/ml of streptomycin. 0.1 ml of the resulting suspension was injected i.m. into the calf of the hind leg using a 20 gauge needle. Following this, animals were randomized into groups of 10 per cage.

Drug and surgical treatment

C. parvum was supplied by the Burroughs-Wellcome Research Labs, Beckenham, England. Each multidose vial contained 7 mg/ml of formalin-killed *C. parvum* in suspension and was stored at 4°C until used. The original suspension was diluted in 0.9% NaCl as required. For local injections 0.1 ml *C. parvum* was given s.c. just above the amputation area of the tumor-bearing leg, near the hip, or into the calf of the left hindleg, 3 days before surgery, on the day of surgery or 5 days thereafter as one single dose (s.c. or i.p.) or as two single doses (s.c. and i.p.) either together, 5 or 8 days apart.

Amputation of the transplanted "primary" tumor was carried out under anaesthesia with 1 mg Nembutal on day 3, 5, 7, 10 or 12 after tumor inoculation. Deaths of animals were recorded daily and autopsies were performed on each to check for the presence of lung metastases and local recurrence at the amputation area. Tumors were measured with calipers and weighed each week.

RESULTS

The effectiveness of radical surgery alone or treatment with *C. parvum* as an adjuvant to surgery was determined according to 3 criteria:

(a) The number of mice surviving to the end of the experiment on day 110 (survivors or cures) (Fig. 1).
(b) The survival time of animals that succumbed (Table 1).
(c) The number of animals with local tumor recurrences (Table 2).

The survival rate of the surgery controls clearly depended on the day of removal of their "primary" tumor. Nineteen out of twenty 3-day-old "primary" tumors were cured by surgery alone. The rate of cures rapidly decreased as the time of surgery was delayed. Benefit from the adjuvant treatment with *C. parvum* was evident when 5 or 7-day-old tumors were amputated. The results at these time points are depicted in Fig. 1.

Nine out of twenty (45%) and 4 out of 20 (20%) of the animals subjected to surgery on day 5 and 7, respectively, survived. In contrast, as high as 9 out of 10 (90%) of the animals survived with adjuvant *C. parvum* treatment on day 5 and 4 out of 10 (40%) with adjuvant *C. parvum* treatment on day 9. *C. parvum* was more effective in animals treated earlier as compared with those treated later. Local treatment at 3 days before amputation yielded the highest percentage of survival, whereas i.p. treatment appeared to be superior to surgery alone when administered on the day of amputation. Combined local and i.p. treatment with *C. parvum* administered prior, subsequent, or at the time of surgery, provided no further improvement in therapeutic response and in some instances showed reduced effectiveness. *C. parvum* had no effect on the survival of mice amputated on day 10 or 12. These groups were therefore excluded from the Fig. 1.

Table 1 shows the median and range of survival time in days of those animals which died before the end of the experiment, in the groups receiving adjuvant treatment with *C. parvum* and surgery controls. In general, for the animals that died, the survival time of the groups receiving adjuvant treatment with *C. parvum* did not differ extensively from the surgery controls.

Table 2 lists the number of animals with tumor recurrence at the area of amputation in relation to the number of animals that died in each group. In most instances there were more local recurrences in the groups that received adjuvant treatment with *C. parvum* than in the surgical controls. In groups treated early (on day −3 or 0) the increase of the rate of local recurrence was more pronounced than in groups treated later (on

Table 1. Median (M) and range (R) of survival times in days after tumor inoculation of animals that died before the end of experiment on day 110, *from the different treatment and control groups*

	Timing of treatment with respect to surgery														
	Day −3				Day 0				Day +5				Surgery only (controls)		
S	T	N/10	M	R	T	N/10	M	R	T	N/10	M	R	N/20	M	R
3	1	1	42		1	1	38		1	2	21	18–24	1	38	
	2	1	38		2	1	95		2	1	10				
	3	0			3	1	31		3	1	24				
	4	1	31		5	3	32	10–47	6	2	43	33–52			
5	1	1	34		1	2	27	26–27	1	4	30	10–96	11	27	7–53
	2	5	42	13–59	2	3	6	6–27	2	5	38	32–48			
	3	1	25		3	4	32	28–35	3	5	35	25–39			
	4	2	36	31–40	5	6	19	6–38	6	5	46	31–74			
7	1	6	29	24–40	1	9	28	24–32	1	8	31	24–49	16	31	24–45
	2	9	32	25–46	2	7	34	26–76	2	7	31	27–47			
	3	9	33	14–63	3	8	30	27–46	3	8	35	25–42			
	4	8	35	27–47	5	9	31	28–35	6	9	28	10–53			
10	1	10	26	24–40	1	10	27	24–34	1	10	27	12–31	19	26	24–38
	2	10	31	11–49	2	10	31	27–46	2	10	30	26–56			
	3	10	31	25–52	3	10	31	26–38	3	10	33	28–38			
	4	10	31	27–39	5	10	31	13–38	6	10	31	24–38			
12	1	10	28	24–32	1	10	29	14–38	1	10	25	13–56	20	26	14–38
	2	10	31	24–33	2	10	30	26–54	2	10	29	13–35			
	3	10	31	27–56	3	10	32	17–35	3	10	31	10–46			
	4	10	31	25–38	5	10	31	13–38	6	10	30	26–39			

S = Day of Surgery. N = Number of mice that died per group out of total. T = Treatment.

Key: 1 = s.c. locally (175 μg in 0.1 ml).
2 = i.p. (350 μg in 0.2 ml).
3 = s.c. locally (175 μg in 0.1 ml) + i.p. (350 μg in 0.2 ml).
4 = i.p. on day −3 + locally 8 days after
5 = i.p. on day 0 + locally 5 days after } same doses as in 1–3.
6 = i.p. on day +5 + locally 5 days before

Table 2. Numbers of animals with local tumor recurrence in relation to the number of the dead animals per group

	Timing of treatment with respect to amputation						No treatment controls
	Day −3		Day 0		Day +5		Co.
A	T	N/10	T	N/10	T	N/10	N/20
3	1	1/1	1	1/1	1	1/2	1/1
	2	0/1	2	0/1	2	0/1	
	3	0/0	3	1/1	3	0/1	
	4	1/1	5	1/3	6	1/2	
5	1	0/1	1	0/2	1	0/4	0/11
	2	0/5	2	0/3	2	0/6	
	3	0/1	3	0/4	3	1/5	
	4	0/2	5	1/6	6	1/5	
7	1	1/6	1	1/9	1	1/8	3/16
	2	5/9	2	3/7	2	2/7	
	3	2/9	3	2/8	3	1/8	
	4	3/8	5	4/9	6	2/9	
10	1	5/10	1	2/10	1	1/10	3/19
	2	2/10	2	2/10	2	0/10	
	3	2/10	3	4/10	3	3/10	
	4	4/10	5	3/10	6	4/10	
12	1	2/10	1	0/10	1	0/10	4/20
	2	2/10	2	1/10	2	2/10	
	3	2/10	3	3/10	3	2/10	
	4	2/10	5	3/10	6	3/10	
Σ	1	9/28 (32%)	1	4/32 (13%)	1	3/34 (9%)	11/67 (16%)
	2	9/35 (26%)	2	6/31 (19%)	2	4/34 (12%)	
	3	6/30 (20%)	3	10/33 (30%)	3	7/34 (21%)	
	4	10/31 (32%)	5	12/38 (32%)	6	11/36 (31%)	

Key of treatment (T) as in Table 1.

day +5). In relation to the day of amputation, an increase in recurrences occurred particularly in animals subjected to amputation on day 7 or 10.

DISCUSSION

The efficacy of *Corynebacterium parvum* in the treatment of metastatic Lewis lung carcinoma was limited to a tumor stage in which only few spontaneous micrometastases had developed. Thus, *C. parvum*, in similarity to other immunopotentiators, appears to be effective in this system only as an adjuvant agent in the treatment of minimal residual disease. The failure of *C. parvum* treatment in other systems may be attributable to the fact that the primary tumor was not removed [20].

The timing of *C. parvum*-treatment with

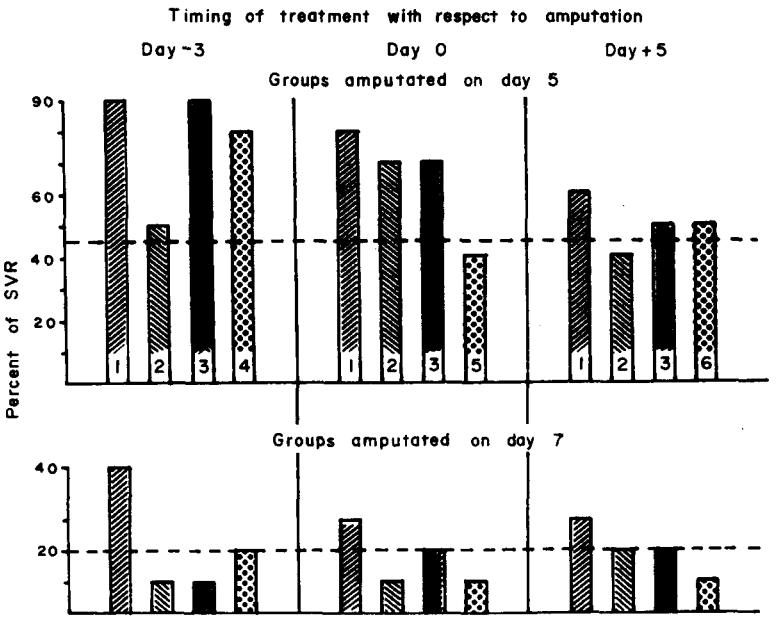

Fig. 1. Percentage of Lewis lung tumor-bearing animals surviving 110 days *after treatment with surgery and* C. parvum. *Each column represents one treatment group comprising* 10 *animals. The dotted line indicates percentage survival of animals not receiving* C. parvum (*surgery controls*). *The surgery controls comprised* 20 *animals per group. Groups amputated on* day 3 *are not shown because nearly all* (19/20) *animals survived following surgery alone and, therefore, no additional effect of* C. parvum *could be measured. Groups amputated on* day 10 *or* 12 *are not shown because no effect of the adjuvant treatment with* C. parvum *on survival was noted.*

Key: 1 = *s.c. locally* (175 μg *in* 0.1 ml). 2 = *i.p.* (350 μg *in* 0.2 ml). 3 = *s.c. locally* (175 μg *in* 0.1 ml) + i.p. (350 μg *in* 0.2 ml).
4 = *i.p. on* day −3 + *locally* 8 days *after*
5 = *i.p. on* day 0 + *locally* 5 days *after* } *same doses as in* 1–3.
6 = *i.p. on* day +5 + *locally* 5 days *before*

respect to surgery also influenced the therapeutic response: *C. parvum* increased survival when administered prior to or at the time of removal of the primary tumor, but resulted in no advantage when administered after primary tumor removal. Several factors could account for the advantage of early immunostimulation: (1) the number of metastatic tumor cells which have to be eliminated through immunostimulation is lower if treatment is started earlier; (2) early administration of the immunoadjuvant could result in accelerated recovery of immune functions possibly impaired by the presence of local and metastatic tumor [21] or by surgery. Local treatment appeared to be somewhat superior to i.p. injections, although only half of the dose was given. Local treatment would provide closer contact between tumor cells and *C. parvum* and could also affect directly the regional lymph node area of the tumor. Such close contact favors the development of immunity, as has been reported [8, 12, 22]. Subcutaneous injections near the tumor site should be distinguished from s.c. injections into other areas, which have been described as being mostly without antitumor effect [22, 23].

The combined administration of *C. parvum* s.c. and i.p. either on the same day or several days apart has no advantage over each as a single dose alone. It would appear that both treatment modalities may even counteract each other [24]. The observation of higher incidence of local tumor recurrence in mice receiving adjuvant *C. parvum* therapy is intriguing, but no clear explanation can be offered. Further investigations are required to obtain additional clarification of the interrelationship of surgery and adjuvant chemotherapy. This includes the role of the dose and duration of treatment as well as combination with other immunoadjuvants or chemotherapeutic agents and detailed investigations of host-tumor immunointeractions. The present system appears to be suitable for study of such questions with a view to clarification of clinical relevance.

REFERENCES

1. H. F. Oettgen, C. M. Pinsky and L. Delmonte, Treatment of cancer with immunomodulators. *Med. Clin. N. Amer.* **60,** 511 (1976).
2. M. T. Scott, *Corynebacterium parvum* as an immunotherapeutic anticancer agent. *Semin. Oncol.* **1,** 367 (1974).
3. J. E. Castro, The effect of *Corynebacterium parvum* on the structure and function of the lymphoid system in mice. *Europ. J. Cancer* **10,** 115 (1974).
4. J. G. Hall and A. R. Moore, The action of local injections of *Corynebacterium parvum* in facilitating the extravasation of activated lymphoid cells. In Corynebacterium parvum—*Applications in Experimental and Clinical Oncology.* (Edited by B. Halpern) p. 112. Plenum Press, New York (1975).
5. B. N. Halpern, A. Prevot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard, Y. Bouthillier and C. Decreusefond, Stimulation de l'activité phagocytaire du système réticuloendothélial, provoquée par le *Corynebacterium parvum. J. Reticuloendothel. Soc.* **1,** 77 (1964).
6. A. Ghaffar, R. T. Cullen, N. Dunbar and M. F. A. Woodruff, Anti-tumor effect *in vitro* of lymphocytes and macrophages from mice treated with *Corynebacterium parvum. Brit. J. Cancer* **29,** 199 (1974).
7. M. Olivotto and R. Bomford, *In vitro* inhibition of tumor cell growth and DNA-synthesis by peritoneal and lung macrophages from mice injected with *Corynebacterium parvum. Int. J. Cancer* **13,** 478 (1974).
8. R. L. Tuttle and R. J. North, Mechanism of antitumor action of *Corynebacterium parvum:* non-specific tumor cell destruction at site of an immunologically-mediated sensitivity reaction to *C. parvum. J. nat. Cancer Inst.* **55,** 1403 (1975).
9. J. E. Castro and T. E. Sadler, Combined surgery and *Corynebacterium parvum* for treatment of a metastasizing tumor in mice. *Brit. J. Surg.* **62,** 22 (1975).
10. B. N. Halpern, Corynebacterium parvum—*Applications in Experimental and Clinical Oncology.* Plenum Press, New York (1975).
11. V. V. Likhite and B. N. Halpern, The delayed rejection of tumors formed from the administration of tumor cells mixed with killed *Corynebacterium parvum. Int. J. Cancer* **12,** 699 (1973).
12. V. V. Likhite, Rejection of tumors and metastases in Fischer 344 rats following intratumor administration of killed *Corynebacterium parvum. Int. J. Cancer* **14,** 685 (1974).
13. St. K. Carter and M. Slavik, A chemotherapeutic perspective on clinical trials with *Corynebacterium parvum.* In Corynebacterium parvum—*Applications in Experimental and Clinical Oncology.* (edited by B. N. Halpern) p. 329. Plenum Press, New York (1975).
14. L. Israel, Report of 414 cases of human tumors treated with Corynebacteria. In Corynebacterium parvum—*Applications in Experimental and Clinical Oncology.* (Edited by B. N. Halpern) p. 389. Plenum Press, New York (1975).
15. R. C. Reed, J. U. Gutterman, G. M. Mavligit, A. A. Burgess and E. M. Hersh, *Corynebacterium parvum*: preliminary report of a phase I clinical and immunological study in cancer patients. In Corynebacterium parvum—*Applications in Experimental and Clinical Oncology.* (Edited by B. N. Halpern) p. 349. Plenum Press, New York (1975).
16. S. R. Humphreys, W. D. DeWys and K. Karrer, A model system for the selection of drugs for chemotherapy of metastasis. *Proceedings of the 5th International Congress of Chemotherapy.* (Edited by K. H. Spitzy and H. Haschek) p. B9/17. Wiener Medizinische Akademie, Vienna (1968).
17. S. R. Humphreys and K. Karrer, Relationship of dose schedules to the effectiveness of adjuvant chemotherapy. *Cancer Chemother. Rep.* **54,** 379 (1970).
18. K. Karrer, S. R. Humphreys and A. Goldin, An experimental model for studying factors which influence metastasis of malignant tumors. *Int. J. Cancer* **2,** 213 (1967).
19. K. Karrer, Formal discussion of W. R. Bruce and H. Lin's paper. An empirical cellular approach to the improvement of cancer chemotherapy. *Cancer Res.* **29,** 2312 (1969).

20. H. WRBA, Comparative effects of various strains of *Corynebacterium parvum* and other prophylactic agents on tumor development in animals. In Corynebacterium parvum—*Applications in Experimental and Clinical Oncology*. (Edited by B. Halpern) p. 314. Plenum Press, New York (1975).
21. T. KURATA and M. MICKSCHE, Correlation of immune response with clinical stage in Lewis lung tumor-bearing mice. *Oncology* **35,** 155–159 (1978).
22. M. T. SCOTT, *Corynebacterium parvum* as a therapeutic antitumor agent in mice. II. Local injection. *J. nat. Cancer Inst.* **53,** 861 (1974).
23. M. T. SCOTT and S. L. WARNER, The accumulated effects of repeated systemic or local injections of low doses of *Corynebacterium parvum* in mice. *Cancer Res.* **36,** 1335 (1976).
24. W. RELLA and K. KARRER, The effect of local and systemic treatment with *Corynebacterium parvum* on the development of lung metastases from Lewis lung carcinoma in mice. *Immunotherapy of Malignant Disease*, Selected papers of an intern. symp. in Vienna (9–12.11.1977), (Edited by H. Rainer) p. 160. Schattauer, Stuttgart (1978).