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Destruction of Regional Lymph Node Metastases of Rat Mammary Adenocarcinoma 13762A by Treatment with *Corynebacterium parvum*[1]

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ABSTRACT

Intratumoral administration of *Corynebacterium parvum* to 13762A tumor-bearing rats on Day 7 of tumor growth, followed by primary tumor excision on Day 20, regularly cured about 40% of the animals and significantly prolonged survival in the remainder. Rats treated by surgery alone on either Day 7 or Day 20 died with metastases to axillary lymph nodes and lungs. Tumor was established in axillary lymph nodes by Day 7. Therefore, intratumoral injection of *C. parvum* on Day 7 destroyed metastases already established at this site. Growth of tumor in axillary nodes of rats treated but not cured by *C. parvum* was significantly slower than growth in untreated rats.

INTRODUCTION

Effective immunotherapy should destroy disseminated tumor cells. This is a critical issue, for if immunotherapy cannot kill established, occult metastases, it offers little advantage over conventional, locally ablative modalities such as surgery or radiotherapy. Immunotherapeutic eradication of disseminated disease has been demonstrated rarely in either human or animal systems (1). Treatment with immune stimulant has been effective against tumors established by prior i.v. injection (2, 5), but there is reason to doubt that such artificial "metastases" are representative of spontaneously disseminated secondary tumors (1). Although some studies demonstrated immunotherapeutic benefits with metastasizing tumors (3, 11, 13), there was no evidence that the micrometastases were established at the time treatment was begun. It was therefore impossible to distinguish between prevention of dissemination (8) or destruction of metastases. In only one system, the guinea pig line 10 hepatoma, was evidence presented that proved that the immune treatment eradicated established, spontaneous metastases (12, 15, 16).

We found that treatment of the 13762A rat mammary adenocarcinoma on the seventh day of tumor growth by i.t.[4] injection of *Corynebacterium parvum*, followed by excision of the primary tumor on Day 20, led to prolonged survivals and significantly fewer deaths due to nodal and pulmonary metastases (7). The primary purpose of this study was to determine whether that result represented an inhibition of tumor cell dissemination or a destruction of established lymph node metastases. Secondary objectives were to establish how soon after i.t. injection of *C. parvum* could the tumor be excised without losing the therapeutic effect and whether injection of *C. parvum* by other routes would be effective. The effects of i.t. *C. parvum* on characteristic growth patterns of primary and nodal metastatic tumor in rats cured by the treatment were compared with similar parameters in rats in which treatment was noncurative.

MATERIALS AND METHODS

Animals. Female Fischer (F344) rats, approximately 6 weeks old, were obtained through the Drug Research and Development Branch, National Cancer Institute, Bethesda, Md. We have established previously that immature rats respond poorly to i.t. *C. parvum* (7); accordingly, rat shipments were held until all rats weighed >120 g.

Tumor. Conditions in our laboratory for the propagation and use of the 13762A rat mammary carcinoma have been described (6, 7). Each passage was examined for bacterial contamination by culture in thioglycolate broth and for macrophage content by microscopic examination of cytocentrifuge preparations stained with May-Grünwald-Giemsa stain. For experiments tumors were initiated by the i.d. injection of 10^6 cells into the right dorsolateral thorax. Tumor size was determined by caliper measurements in 3 dimensions, and the GMD was calculated (7). On the seventh day of growth, tumors were measured and animals were assorted sequentially into groups on the basis of decreasing tumor size. Each group contained 10 rats unless otherwise indicated. The size of primary and axillary nodal tumors was determined on the 20th day of growth. Primary tumors were excised while rats were under ether anesthesia. Statistical comparisons of survival time were made with the Mann-Whitney *U* test. Significance of cure frequency was established with Fisher's exact probability test or the χ^2 test. Dead animals were examined at autopsy.

C. parvum suspensions were obtained through the courtesy of Dr. John Whisnant, Burroughs-Wellcome Research Laboratories, Research Triangle Park, N. C. In all the experiments reported here, the dose was 1500 μg (dry weight) per 0.1 ml per animal. The method of i.t. *C. parvum* injection was described previously (7).

RESULTS

Destruction of Established Axillary Nodal Metastases by *C. parvum* i.t. The incidence of residual tumor was

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[4] The abbreviations used are: i.t., intratumor; i.d., intradermal; GMD, geometric mean diameter.

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compared in rats that received surgery alone on Day 20, or surgery on Day 20 plus *C. parvum* i.t. on Day 7. A third group had primary tumor excision on Day 7. This latter group was intended to demonstrate the proportion of rats in which tumor cells had already escaped the primary tumor by Day 7, *i.e.*, those in which the 7-day primary tumor excisions were noncurative. A total of 7 such experiments were performed. The results (Table 1) demonstrated that surgery alone on Day 7 cured less than 5% of rats thus treated. Surgery on Day 20 caused similar results; only 3% of rats thus treated were cured. These results prove that, by 7 days of tumor growth, most rats already had disseminated metastases. *C. parvum* given on Day 7, followed by primary tumor excision on Day 20, significantly reduced the incidence of deaths. Clearly, *C. parvum* given at 7 days destroyed established nodal metastases and did not simply prevent subsequent dissemination.

Duration of *C. parvum* i.t. Residence Required to Improve Survival. An experiment was designed to determine how long *C. parvum* must reside in the primary tumor to destroy nodal metastases. Rats with 7-day-old tumors were either left untreated or given i.t. injections of *C. parvum*. Three hr after injections were completed, primary tumors were excised from 10 of the *C. parvum*-injected rats and from 10 of the uninjected control rats. The excision of *C. parvum*-injected and uninjected tumors was performed on other rats on Days 10, 14, 16, and 20 of tumor growth. *C. parvum* injections (Chart 1) improved survival of all groups irrespective of the day of surgery (7 days, $p < 0.05$; 10 days, $0.05 > p > 0.01$; 14 days, $p < 0.002$; 16 days, $p < 0.05$; 20 days, $p < 0.05$). We concluded that *C. parvum* need not be in the primary tumor for more than 3 hr for the successful eradication of metastases.

Importance of the Site of *C. parvum* Injection. In our previous study (7) *C. parvum* was given only i.t. The importance of the site of *C. parvum* injection was not previously tested in this system. Rats with 7-day-old tumors received injections at various sites: (*a*) i.t.; (*b*) i.d. on the contralateral side; (*c*) i.d. on the ipsilateral side between the primary tumor and the adjacent axillary lymph nodes; (*d*) i.v. via the tail vein; or (*e*) no *C. parvum;* instead, the primary tumor was excised. The results (Chart 2) showed a strong effect (both in the prolongation of survival and in the 60% cures) of *C. parvum* given i.t. and a lesser but significant effect of

Table 1

Destruction of residual tumor in rats treated by primary tumor excision at either 7 or 20 days with i.t. injections of C. parvum given at 7 days of tumor growth and followed by primary excision at 20 days

	Treatment groups[a]		
Experiment	Primary tumor excised at 20 days	Primary tumor excised at 7 days	*C. parvum* i.t. given at 7 days and primary tumor excised at 20 days
1	7/8	10/10	4/10[b]
2	11/11	12/12	8/11
3	11/11	10/11	5/11[b]
4	10/10	10/10	5/10[b]
5	11/11	11/11	9/11
6	11/11	10/10	6/11[b]
7	10/11	8/11	8/11
Totals	71/73 (97.3)[c]	71/75 (95)	45/75[b] (60)

[a] Number of rats dying with residual tumor/total rats.
[b] Significantly less than total of 7-day primary excision groups; $p < 0.05$, χ^2 test.
[c] Numbers in parentheses, percentages.

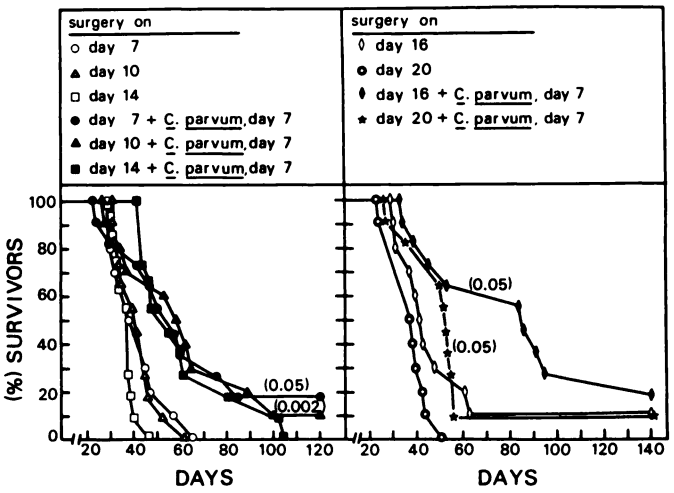

Chart 1. Survival of 13762A tumor-bearing rats treated with 1500 μg *C. parvum* i.t. on Day 7 followed by excision of primary tumor on Days 7, 10, 14, 16, or 20. *Numbers* in *parentheses* are greater than the probability that prolongation of survival relative to the group receiving surgery alone was due to chance (Mann-Whitney *U* test).

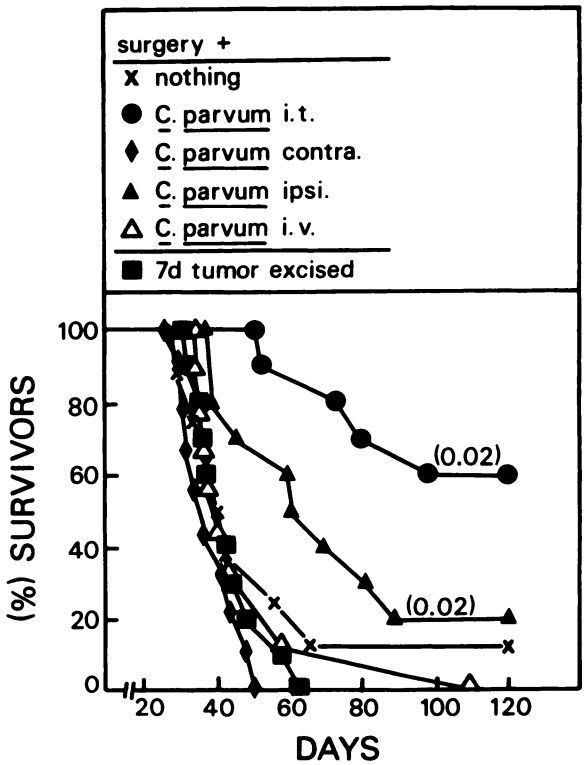

Chart 2. Survival of 13762A tumor-bearing rats treated with 1500 μg *C. parvum* on Day 7. Injections were given i.t., i.d. contralaterally (*contra.*), i.d. ipsilaterally (*ipsi.*) between the primary tumor and the axilla, or i.v. One group of rats received only primary tumor excisions on Day 7 (*7d*), and all other groups received primary tumor excisions on Day 20. *Numbers* in *parentheses* are greater than the probability that prolongation of survival relative to the group receiving surgery alone on Day 20 was due to chance (Mann-Whitney *U* test).

C. parvum given ipsilaterally between the primary tumor and the lymph node. *C. parvum* given either i.d. contralaterally or i.v. was ineffective. Primary tumor excision at 7 days did not affect mortality or survival. Thus, it was not necessary for *C. parvum* to be given directly into the tumor. Although it was most effective i.t., it significantly prolonged survival when given i.d., presumably into the lymphatic drainage from the primary tumor.

Comparison of the Effects of *C. parvum* i.t. on Primary and Axillary Tumor Size in Treated and Control Rats. For identification of factors associated with a successful outcome of *C. parvum* treatment, a series of experiments was conducted which all included at least the same 2 groups. One group received only primary tumor excision on Day 20 (the usual negative control). The second group received 1500 μg *C. parvum* i.t. on Day 7 followed by primary tumor excision on Day 20 (the usual treatment group). Results from those experiments were used to test several hypotheses to explain the variability in response to the usual treatment.

One possible explanation for the variable effectiveness of *C. parvum* i.t. might be that treated rats had smaller primary tumors at the time of *C. parvum* injection (7 days) than did the untreated rats and that the smaller tumors were more susceptible to cure. The sizes of primary tumors on Day 7 in control and treated groups were compared. The results (Table 2) demonstrated that *in* the primary tumors into which *C. parvum* was injected, tumor size distribution was identical with those of the untreated tumors. These data validated our strategy of assigning rats to experimental groups. In this method animals in an experiment were not allocated to the various groups until the seventh day, when primary tumors were measured and the animals were ranked on the basis of primary tumor size. (Axillary tumors were not palpable at this time.) They were then assorted consecutively into groups. This strategy should assure an equivalent distribution of tumor size in all groups, and the data presented here indicate that the assumption was correct. Therefore, any subsequent differences in tumor growth cannot be attributed to an initial bias in allocation of animals to experimental groups.

An obvious beneficial effect of *C. parvum* i.t. would be to inhibit growth of primary and/or axillary tumor before surgery. We compared the tumor sizes on Day 20, the time of primary tumor excision. The results (Table 3) clearly indicated that *C. parvum* treatment slightly reduced the growth rate of the primary tumor and strongly inhibited the growth of the axillary metastases.

Comparison of Primary and Axillary Tumor Sizes and Body Weights in Rats Cured or Not Cured by Treatment with *C. parvum* i.t. *C. parvum* regularly cured some treated rats. The cure frequency was variable, and factors predisposing or associated with cure were unknown. The *C. parvum* treatment groups of a series of experiments were separated into "cured" and "not cured" subgroups, and a number of possible factors that might have contributed to cure were assessed.

It was theoretically possible that of all *C. parvum*-treated rats, those with smaller primary tumors at the time of i.t. injection might be cured more readily than those with larger tumors. We compared primary tumor size on Day 7, the day of i.t. *C. parvum* injection, in rats cured or not cured by this treatment (Table 4). The 2 subgroups had identical tumor sizes so the possibility was excluded.

In attempts to identify factors that contribute to prognosis and that might permit early identification of rats that would be cured in the *C. parvum*-treated group, we compared the primary and axillary tumor sizes on Day 20 in those treated rats cured with tumor sizes in those not cured by treatment (Table 5). The results revealed a slight but significantly greater inhibition of primary tumor growth in the cured rats than in the rats not cured by the *C. parvum* treatment. The most striking effect was an almost total absence of axillary node metastases at 20 days in the rats destined for cure.

Table 2

Comparison of primary tumor size at 7 days in rats assigned to experimental groups[b]

Treatment group	Primary tumor GMD (mm)	No. of rats
Untreated	3.6 ± 0.2[b]	102
C. parvum-treated	3.6 ± 0.3	104

[a] Pooled data of 10 consecutive experiments.
[b] Mean + S.E.

Table 3

Comparison of primary and axillary tumor sizes at 20 days in untreated rats and rats treated with C. parvum i.t.[a]

Treatment group	Tumor GMD (mm)		No. of rats
	Primary	Axillary	
Untreated	16.0 ± 0.5[b]	9.3 ± 0.6	102
C. parvum-treated	13.3 ± 0.5[c]	3.1 ± 0.6[c]	104

[a] Pooled data of 10 consecutive experiments.
[b] Mean ± S.E.
[c] $p < 0.001$ compared with untreated group; Student's *t* test.

Table 4

Comparison of primary tumor size at 7 days of rats either cured or not cured after C. parvum i.t. on Day 7 and surgery on Day 20[a]

Results of treatment	Primary tumor GMD (mm)	No. of rats
Cured	3.3 ± 0.3[b]	39
Not cured	3.7 ± 0.2	65

[a] Pooled data of 10 consecutive experiments.
[b] Mean ± S.E.

Table 5

Comparison of primary tumors or axillary metastases at 20 days in rats either cured or not cured after C. parvum i.t. on Day 7 and surgery on Day 20[a]

Results of treatment	Primary tumor GMD (mm)	Axillary tumor GMD (mm)	No. of rats with axillary tumor/total rats
Cured	11.6 ± 0.9[b]	0.1 ± 0.1	1/38
Not cured	14.5 ± 0.7[c]	4.9 ± 0.8[c]	28/66

[a] Pooled data of 11 consecutive experiments.
[b] Mean ± S.E.
[c] $p < 0.001$ compared with cured group; Student's *t* test.

Table 6

Comparison of size of axillary metastases at 20 days in rats dying after treatment with surgery alone at 20 days with that of rats dying after treatment with C. parvum i.t. on Day 7 followed by surgery on Day 20[a]

Treatment group	Axillary metastases GMD (mm)	No. of rats
Untreated	9.3 ± 0.6[b]	102
C. parvum i.t. (not cured)	4.9 ± 0.8[c]	64

[a] Pooled data of 10 consecutive experiments.
[b] Mean ± S.E.
[c] $p < 0.001$ compared with untreated group; Student's *t* test.

Table 7

Comparison of body weights in rats either cured or not cured after treatment with C. parvum i.t. on Day 7 and surgery on Day 20[a]

Results of treatment	Body wt GMD (g)	No. of rats
Cured	138 ± 3[b]	23
Not cured	134 ± 2	41

[a] Pooled data of 6 consecutive experiments.
[b] Mean ± S.E.

The incidence of metastases reported here at 20 days does not represent the final or interim incidence. Almost all rats not cured usually died with axillary metastases. A very small number of the cured rats experienced transient growth of axillary metastases with eventual complete regression. However, the results clearly indicated that the presence of axillary metastases at 20 days was a useful prognostic indicator for individual rats and was certainly more reliable than primary tumor size.

Although growth of axillary metastases was suppressed in rats that were cured by the *C. parvum* treatment, there was no information yet available on whether the axillary metastases were inhibited in rats that were not cured by the treatment. To answer this question we compared the size of axillary metastases in the *C. parvum*-treated-but-not-cured group with that in the group treated by surgery alone at 20 days. The results (Table 6) indicated that even in rats not cured by the *C. parvum* treatment there was significant inhibition of axillary metastases.

The last factor examined was the influence of rat body weight. Immature rats did not respond to *C. parvum* i.t., whereas older rats had a significantly improved survival time and some were cured (7). All mature rats in our subsequent experiments weighed >120 g, but some have been considerably heavier. To determine whether host body weight above this threshold was a factor in the outcome of *C. parvum* treatment, we compared the body weights of cured and not cured *C. parvum*-treated rats (Table 7). The results indicated that body weight in excess of 120 g was not a factor associated with cure.

DISCUSSION

The major task of immunotherapy is to cure a tumor that has metastasized beyond the reach of surgery or radiotherapy. However, as has been emphasized by Alexander (1), few animal models of immunotherapy have dealt with disseminated disease. Experimental immunotherapy has been applied most frequently to tumors that rarely if ever metastasize (10) or to "metastases" established artificially by i.v. injection of tumor cells (2, 5). In some metastasizing tumors treated by immunotherapy, metastasis was reduced, but it was not clear whether the treatment simply prevented dissemination (8) or actually destroyed preexistent metastases (3, 11). In another study (9) pulmonary metastases were reduced but not eliminated by postsurgical immunotherapy. Total eradication of metastases by immunotherapy has been achieved decisively in only one animal model. Treatment of the guinea pig line 10 hepatoma on Day 7 by i.t. injection of *Bacillus Calmette-Guérin* eradicated nodal metastases that antedated treatment (12, 15, 16). The present study permits similar, firm conclusions for the therapeutic effect of *C. parvum* i.t. in the 13762A rat mammary adenocarcinoma. The regional lymph node metastases were well established prior to initial treatment. *C. parvum* injected i.t. cured significant numbers of the rats and prolonged the survival of the remainder. To the best of our knowledge, the line 10 hepatoma and the 13762A carcinoma are now the only model systems in which cure of spontaneous metastases by immunotherapy has been demonstrated unequivocally.

It was not necessary for *C. parvum* to be injected i.t. Injections between the regional lymph node and the primary tumor prolonged survival but were not as effective as the i.t. injection. This result, as well as the brief duration of *C. parvum* i.t. residence required, strongly suggested that the primary tumor was not an essential component of *C. parvum* immunotherapy in this system. Failure of i.v. or contralateral i.d. *C. parvum* injections to affect tumor growth argues for an important role of the regional lymphatics in channeling *C. parvum* to the nodal metastases.

By Day 20 *C. parvum*-injected primary tumors and associated axillary node metastases were smaller than the controls. The most striking effects of *C. parvum* were found when *C. parvum*-cured rats were directly compared with *C. parvum*-treated rats in which the treatment was not curative. Virtually no axillary metastases were detectable at 20 days in the cured rats. Growth rate of axillary node metastases was retarded even in those rats that were not cured by *C. parvum* treatment. Therefore, therapeutic benefits in this system were not the result of an "all-or-none" mechanism; *i.e.*, the outcome was dependent upon quantitative rather than qualitative factors. It follows that improvements in therapeutic benefits may result from appropriate adjustment of variables rather than a major alteration in approach.

The 13762A rat mammary adenocarcinoma has distinct advantages over the guinea pig line 10 hepatoma that commend it as an important and relevant model system for tumor immunotherapy. We have studied the pathology of lethal tumor growth in both systems. Guinea pigs that succumbed to the hepatoma usually developed massive axillary and mediastinal lymph node metastases but pulmonary parenchyma and other organs were rarely involved (4). In contrast, extensive spontaneous metastases, almost always to lungs and occasionally to other organs, were a common finding in rats dying with postsurgical 13762A tumor (6, 7). Additional experiments to characterize the

development of pulmonary metastases and the effect of immunotherapy on these metastases are now in progress. Radical surgery on Day 7, including extensive axillary lymph node dissection, was rarely curative for the 13762A (J. W. Kreider, unpublished data). An additional advantage of the rat tumor system is that F344 rats are comparatively cheap and readily available. The expense and limited availability of strain 2 guinea pigs have restricted the study of the line 10 hepatoma to a few laboratories.

That immunotherapy can cure established metastases is indeed good news. Our results demonstrate that this achievement is not unique to the line 10 tumor, and they thereby raise the hope that comparable effects may be attained in other experimental systems and eventually in human tumors. In the current climate of pessimism concerning the practical impact of immunotherapy (1, 14), this should reaffirm that immunotherapy has potential for treatment of residual, disseminated cancer.

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