ABSTRACT

We studied the effects of intratumor injection of Corynebacterium parvum vaccine on the survival of 13762A tumor-bearing rats. Vaccine injection of established (7-day-old) tumors produced dose-related prolongation of survival and cured some animals. Although 30 to 40% of the vaccine-injected primary tumors regressed, recurrences and continued growth of metastases ultimately killed one-fourth of the regressors. Rats given 1500 µg of C. parvum intratumorally at 7 days, with or without later primary tumor excision at 20 days were cured at a rate of 10 to 40%. Repeated injections improved the results (60%). C. parvum injections delayed until 12 and 17 days were ineffective. Cured rats were immune to rechallenge with 13762A tumor.

MATERIALS AND METHODS

Animals and Tumors. Female F344 rats 5 to 6 weeks old were obtained from the Frederick Cancer Research Center, Frederick, Md. We have previously described the origin and conditions of propagation and use of the 13762A rat mammary adenocarcinoma (3). Freedom from contamination was documented by culture of each passage in thioglycolate broth. Cyto centrifuge preparations stained with Giemsa were also obtained at each passage. We have found that when macrophage numbers in the ascites are >25%, poor growth of the tumor cells results. The R3230AC tumor was obtained from Dr. Paul Fritz, Milton S. Hershey Medical Center. Tumors were established by the i.d. injection of 10^6 cells on the right dorsolateral thorax. The i.d. tumors were measured with a caliper in 3 dimensions and tumor size was calculated as the geometric mean diameter (GMD):

GMD = \frac{1}{3}d1 \times d2 \times d3 (mm)

Prior to i.t. injection of C. parvum on the 7th day of tumor growth, tumors were measured (usually about 5 mm) and the animals were sequentially assorted into experimental groups on the basis of decreasing tumor size. Each group contained 10 rats unless otherwise specified. Statistical comparisons of survival times were made with the Mann-Whitney U test. Significance of the frequency of cures was evaluated with the Fisher’s exact probability test.

Immune Stimulant. Stock suspensions of killed C. parvum (7 mg dry wt/ml) vaccine were supplied by Dr. John Whisnant, Burroughs-Wellcome Research Laboratories, Research Triangle Park, North Carolina. For tumor injection, a 25-gauge needle was passed through the skin about 5 mm from the tumor and directed into the bottom of the tumor. A total of 0.1 ml of phosphate-buffered saline (0.68% NaCl, 0.17% NaHPO4, and 0.02% KH2PO4, pH 7.4) containing the C. parvum dose was delivered in approximately equal amounts into the 4 quadrants of the tumor.

RESULTS

Comparison of i.t. C. parvum Treatment of 13762A in Young and Old Rats. During the course of experiments, we...
observed considerable variability in the response of different shipments of rats to treatment with i.t. injection of C. parvum. The results suggested that host age might be an important factor. To test this hypothesis, tumors were initiated (10⁶ cells i.d.) in 2 groups of 10 rats held previously for either 4 weeks (Group A, approximate age 9 to 10 weeks; average body weight 120 g) or 1 week (Group B, approximate age 5 to 6 weeks; average body weight 83 g). A third group of 20 rats (Group C) from the same batch as Group B was held for an additional 4 weeks and then given tumor cells. On the 7th day of tumor growth, the tumors of 10 rats were infiltrated with 1500 μg of C. parvum in 0.1 ml of phosphate-buffered saline.

Survival of both groups of rats that had been held for 4 weeks was significantly prolonged by i.t. injection of C. parvum, and 3 were cured. In contrast, the younger rats did not respond to treatment (Chart 1). The results indicated that an optimal response to i.t. injection of C. parvum required the use of rats held in our facilities for at least 1 month prior to use in experiments. Although the factor(s) responsible for this effect was not clear, all subsequent experiments have been conducted with the older rats.

Effect of C. parvum Dose on the Treatment of 13762A Tumors. In 2 independent experiments (Charts 2 and 3), 7-day-old tumors were infiltrated with various doses of C. parvum. In one experiment (Chart 2), doses of 0, 7, 70, 350, 700, and 1500 μg of C. parvum were used; in the other experiment (Chart 3), the doses were 0, 1500, 3000, and 6000 μg. Many (10 to 50%) of the established primary tumors underwent partial or complete regression. For about 3 weeks after C. parvum treatment, even those tumors which later completely regressed were grossly indistinguishable from uninjected tumors. The regressions were not always curative, since in some rats (25% of regressors) the primary tumor subsequently recurred or nodal and pulmonary metastases developed and killed the hosts. In both experiments survival was significantly prolonged by all treatment doses. Significant numbers of rats were cured by 350 μg (Chart 2) and 3000 and 6000 μg (Chart 3). The effects obtained were dose proportional, except for 350 μg. Since this dose has not been used in other experiments, we do not know whether the result was fortuitous. The 2 highest doses (3000 and 6000 μg) are amounts impractical for routine use, so most of our subsequent studies have used 1500 μg.

Resistance to Challenge of Rats Cured of 13762A Tumors by i.t. Injection of C. parvum. In the 2 preceding experiments, a total of 22 rats were cured by 13762A tumors by treatment with i.t. injection of C. parvum. These rats and previously untreated rats were challenged with 1 × 10⁶ 13762A tumor cells i.d. on the side contralateral from the original tumor site (Table 1). None of the 22 cured rats developed tumors, but all 20 of the control rats died from tumor. The 13762A-resistant rats and 10 previously untreated rats were then challenged with 1 × 10⁶ trypsin-dissociated R3230AC tumor cells. This syngeneic mammary adenocarcinoma is biologically similar to the 13762A tumor (2) but is apparently antigenically distinct. Both the untreated and 13762A-resistant rats developed progressively growing R3230AC tumors, with no difference in growth rates.

Treatment of 13762A Tumors with i.t. Injection of C. parvum followed by Surgery. These experiments were attempts to improve the results of primary tumor excision by presurgical i.t. C. parvum treatment. The general experimental design consisted of C. parvum infiltration of 7-day-old 13762A tumors, followed by excision of the tumor on the 20th day of tumor growth. The variables studied in these 3 experiments were C. parvum dose, delay of C. parvum treatment, and repeated C. parvum injections.

In the first experiment (Chart 4), animals treated by surgery alone all died by Day 90. Presurgical injection (on Day 7) of 3000 or 1500 μg of C. parvum resulted in 3 cures in each group, whereas only 1 rat was cured by 700 μg of C. parvum (individual groups not significant). Survival was...
C. parvum Treatment of 13762A Tumor

Chart 3. Survival of 13762A tumor-bearing rats treated with various doses (1500 to 6000 µg) of C. parvum alone given on the 7th day of tumor growth. Numbers in parentheses are greater than the probability that prolongation of survival relative to no treatment group was due to chance (Mann-Whitney U test).

Table 1
Resistance to tumor cell challenge of rats cured of 13762A tumor by i.t. C. parvum

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Challenge tumor</th>
<th>Tumor incidencea</th>
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<tbody>
<tr>
<td></td>
<td>13762A</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>R3230AC</td>
<td>8/8</td>
</tr>
<tr>
<td>1</td>
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<td>15/15</td>
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<tr>
<td></td>
<td>13762A</td>
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<tr>
<td></td>
<td>R3230AC</td>
<td>14/14</td>
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<tr>
<td>2</td>
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</tbody>
</table>

a Number of rats developing tumor at challenge site/number of rats challenged.

not significantly prolonged by any dose.

In the second experiment (Chart 5), animals of the control group (tumor excision Day 20, no C. parvum) were all dead by Day 100. C. parvum (1500 µg) given on Day 7 significantly prolonged survival of the group, including 3 cures (P < 0.02, Mann-Whitney U test). However, the same dose of C. parvum given i.t. on either Day 12 or Day 17 did not prolong survival.

Control rats in the third experiment (Chart 6) were all dead by Day 100. Presurgical i.t. injection of C. parvum on Day 7 cured 4 of 10 rats (P < 0.04) and prolonged survival (P < 0.002). The other 2 treatment schedules (presurgical C. parvum injections on Days 7 and 12, or on Days 7, 12, and 17), each cured 6 of 10 rats (P < 0.04) and prolonged survival. The repeated injection protocols combined with surgery produced the highest frequency of cures of any treatment studied, but the difference from the single injection group in this experiment was not significant.

Morphology of C. parvum-injected Tumors. 13762A tumors injected with C. parvum on the 7th day of tumor growth were excised on the 20th day. Sections of these tumors revealed only moderate infiltration by mononuclear leukocytes, principally small lymphocytes. The infiltrate of the C. parvum-injected tumors was slightly more intense than the infiltrate of control, un.injected tumors. Macrophages were occasionally present. Mast cells appeared primarily in the adjacent dermal tissues and were not more numerous in the injected tumors. We did not find tumor cell necrosis in association with the leukocytic infiltrate.

DISCUSSION
This is the first systematic study in the 13762A system of variables, such as host age, dose, timing, and repeated injections, which influences the success of i.t. immunotherapy with C. parvum. The administration of C. parvum injected i.t. on the 7th day of 13762A tumor growth strongly inhibited the growth of primary tumors and induced partial and complete regressions. The development of both nodal and pulmonary metastases was often prevented by i.t. injection of C. parvum and some tumor-bearing rats were cured. The cured rats were strongly and specifically resistant to tumor cell challenge. We do not know all of the
numbers in parentheses are greater than the probability that prolongation of survival relative to no treatment group was due to chance (Mann-Whitney U test).**

Factors that determined good or poor results in this system but some conclusions can be drawn from this study.

The age of the tumor-bearing rats was critical. Young rats did not respond to i.t. injection of *C. parvum* whereas older rats did. The shift from unresponsiveness to responsiveness occurred when rats were held for 1 month prior to experiments. Factors responsible were not defined. Although immunological maturation was an attractive possibility, other nonimmunological factors (hormones, metabolism, environmental adaptation, etc.) cannot be excluded.

The frequency of cure and the prolongation of survival was proportional to the *C. parvum* dose injected. The cure frequency of 1500 µg of *C. parvum* given i.t. in 6 experiments varied from 10 to 40% (average 25%). In further experiments practical considerations limited the dose of *C. parvum* to 1500 µg. For maximal effect, treatment had to be repeated doses of 1500 µg of *C. parvum* i.t. given on Day 7, or Days 7 and 12, or Days 7, 12, and 17. Tumors were subsequently excised on Day 20. Numbers in parentheses are greater than the probability that prolongation of survival relative to no treatment group was due to chance (Mann-Whitney U test).

In other animal models, i.t. injection of immune stimulants has produced effects varying from enhanced growth (4) to complete suppression (8). In one interesting report, even histologically similar rat tumors differed in response to i.t. immune stimulants (7). In a system related to ours (5), *C. parvum* to 1500 /¿g. For maximal effect, treatment had to be repeated doses of 1500 ^9 of *C. parvum* i.t. given on Day 7, or Days 7 and 12, or Days 7, 12, and 17. Tumors were subsequently excised on Day 20. Numbers in parentheses are greater than the probability that prolongation of survival relative to no treatment group was due to chance (Mann-Whitney U test).

Guérin regularly produced complete regression of primary tumors and suppression of regional lymph node metastases (8). Although primary tumor regression could be the consequence of local, nonspecific immunity, the simultaneous regression of primary and regional lymph node metastases argued convincingly for augmentation of tumor-specific resistance. Certainly, cured animals possessed strong tumor rejection immunity. Although this system has provided much essential information, comparison with other, biologically unrelated models would permit identification of those generalizations that might be applicable to human i.t. immunotherapy.

What mechanism(s) might be involved in the beneficial effects of i.t. injection of *C. parvum* in the 13762A system? Although we have not specifically studied the mechanisms of the antitumor effects of i.t. injection of *C. parvum*, 2 possibilities appear most likely: (a) An immune response may develop against the *C. parvum*, and tumor cells could be killed nonspecifically as innocent bystanders at the injection site; or (b) i.t. injection of *C. parvum* may produce an augmented systemic, tumor-specific immune response. The 2 processes are not mutually exclusive, and a combination of the 2 would resemble the guinea pig line 10 model. A local, nonspecific reaction could explain both the complete regression of the primary tumor and its occasional subsequent recurrence following elimination of the injected *C. parvum*. Nodal and pulmonary metastases might be affected only if the *C. parvum* reduced the probability of metastases through prior nonspecific destruction of the primary tumor, or if the nonspecific destruction were extended by dissemination of *C. parvum* with tumor cells. On the other hand, if i.t. injection of *C. parvum* stimulated a tumor-specific immune response, primary tumor regressions and cure of metastases could result. If cures are the result of augmented immunity, then recurrent primary tumors and persistent metastases might represent tumor sublines that had escaped rejection by any of a number of mechanisms. Present data are not adequate to define the relative contributions of nonspecific or specific immune responses as possible mechanisms in cure of 13762A by i.t. injection of *C. parvum*.

**REFERENCES**

Immunotherapy of an Established Rat Mammary Adenocarcinoma (13762A) with Intratumor Injection of Corynebacterium parvum

John W. Kreider, Gerald L. Bartlett, Dallas M. Purnell, et al.


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