Experimental Cancer Immunotherapy: Comparison of Tumor Rejection in F344 Rats Given Live Mycobacterium bovis (Strain BCG) and Killed Corynebacterium parvum

Vilas V. Likhite

SUMMARY—F344 rats received grafts of syngeneic 13762 mammary adenocarcinoma cells previously admixed with either living BCG or killed Corynebacterium parvum administered sc or intradermally (id). Animals given id transplants of tumor cells admixed with either BCG or killed C. parvum exhibited tumor growth for an average of 10 days, then regression in size and rejection of the tumor nodules. Lesions were found in rats given sc transplants of tumor cells admixed with the killed microorganism for an average of 13 days with the same results. When live BCG was added to the sc transplants, accelerated rates of tumor growth and early death were noted, compared with the group receiving tumor cells alone sc. Suppressed rates of tumor growth and prolonged survival were observed in the groups receiving id inoculations of tumor cells followed by treatment with killed C. parvum administered weekly ip or id 1 cm away and around the growing tumor. On the other hand, weekly treatment of BCG injected either ip or id 1 cm away and around the growing tumor resulted in accelerated rates of tumor growth and early death. Animals exhibiting C. parvum or BCG-mediated tumor rejection displayed tumor-specific protection to sc challenge injections of the cell line initially used, but they died with growing tumors and metastases when challenged with tumor cells of an antigically different line syngeneic to F344 rats. Microscopic examination of histologic sections of tumors formed from id inoculations of tumor cells admixed with either BCG or killed C. parvum revealed a nonspecific infiltrate of macrophages and polymorphonuclear leukocytes in the tumor, whereas sections of tumors formed from sc grafts of cells admixed with killed C. parvum revealed a specific organized infiltrate of mostly macrophages around the tumor follicles.—J Natl Cancer Inst 56: 985–989, 1976.

Optimal results with the use of living Mycobacterium bovis (strain BCG) in experimental cancer immunotherapy were first reported by Zbar and co-workers (1), who observed that BCG totally prevented the growth of tumor cells in recipients, when it was admixed with living tumor cells before they were inoculated intradermally (id) [the id site promotes tumor growth and regression (2)]. Furthermore, intratumor infection with BCG into growing intradermal tumor nodules also resulted in regression and complete rejection (3, 4). A completely different phenomenon was observed in animals receiving sc injections (the sc site promotes early dissemination) of tumor cells as an inoculum containing killed Corynebacterium parvum. These animals exhibited expected tumor growth (at the injection site) for approximately 2 weeks. Thereafter, tumor-specific rejection of the growing tumors occurred in all the animals (5). Subsequent studies revealed that intratumor-permeating injections of killed C. parvum also resulted in rapid rejection of subcutaneously growing tumors in syngeneic animals (6, 7), and the conferred immune response was effective in protecting these animals against progressive growth of potentially lethal metastases present at the time of therapy. The studies presented here, in which living BCG and killed C. parvum are used, compare the rejection of tumors syngeneic to F344 rats.

MATERIALS AND METHODS

Rats.—Female 40- to 50-g F344 rats were obtained from the Charles River Breeding Laboratories (Wilmington, Mass.) and kept under normal laboratory conditions. C. parvum.—Preparation of killed C. parvum were obtained from Institut Merieux (Lyon, France). The method of preparation was described in (5).

Histologic sections.—Formol (10%) in Hanks’ solution was used as a fixative. Using standard histologic techniques, we prepared sections for microscopic examination with hematoxylin-eosin and Giemsa stains.

Evaluation of tumor growth.—After transplanting the tumor cells, we inspected the animals daily and measured the palpable tumors with a caliper twice weekly. Tumor size was expressed as the average of two diameters (perpendicular to each other). Animals in the control and experimental groups were compared at the end of the experiment, and the differences between the groups were tested for significance by the paired t-test.

b) DMB 14 mammary adenocarcinoma, a dimethylcholanthrene-induced carcinoma emerging in an F344 female rat, has been passed serially without loss of characteristics. The 100% lethal sc dose in recently weaned animals was 104–106 tumor cells, and the animals died with growing tumors and metastases in 50–70 days.

b) The 13762 mammary adenocarcinoma syngeneic to F344 rats was obtained from the Mason Research Institute (Worcester, Mass.) and passed serially in syngeneic females without loss of its characteristics, which were described (7). Sensitization of rats to an irradiated tumor of one tumor cell line results in protection against subsequent injections of tumor cells only of that line.

Tumor implantation.—Using aseptic techniques, we removed tumor tissue from recently killed donor animals, cut it into pieces of various sizes, and trimmed connective tissue and any grossly apparent necrotic or hemorrhagic material from each piece. The glistening portions were then cut into 1- to 2-mm3 slices. Suspensions of tumor cells in medium 199 were obtained by mild manipulation of the tumor slices on surgical tantalum gauze; they were washed 3 times (10X vol medium 199; 500×g for 10 min) and then resuspended in the medium. The final suspension was allowed to set for 2 minutes to sediment the larger clumps of cells, and then the supernatant was removed and readjusted to a cell count of approximately 2.5×105 cells/ml.

Sixty previously unsensitized rats were separated into groups of 10 each. The size of each tumor-cell inoculum dose was 5×105 13762 cells injected in a volume of

1 Received June 20, 1975; accepted November 7, 1975.
2 Thorndike Memorial Laboratory, Harvard Medical Unit; Harvard Medical School, Building E2, 25 Shattuck St., Boston, Mass. 02115.
3 Junior Faculty Fellow of the American Cancer Society.
0.2 ml. All rats of a group each received one of the following: 1) 13762 cells id; 2) 13762 cells sc; 3) 13762 cells admixed with 10⁶ BCG organisms id; 4) 13762 cells admixed with 10⁵ BCG organisms sc; 5) 13762 cells admixed with 1 mg C. parvum id; 6) 13762 cells admixed with 1 mg C. parvum.

RESULTS

The groups of rats receiving id inoculations of tumor cells mixed with killed C. parvum or live BCG exhibited minimal amounts of tumor growth for about 10 days; then the tumors disappeared in all animals (text-fig. 1). The group receiving sc inoculations of tumor cells admixed with C. parvum had normal rates of tumor growth for approximately 13 days; all the tumors subsequently regressed and disappeared. The group receiving tumor cells admixed with BCG sc, when compared with the group receiving only tumor cells, showed slightly enhanced rates of neoplastic growth and died with developing tumor and metastases in 30–40 days. Those animals receiving the 13762 cells alone sc died in 40–52 days with growing tumor and metastases, whereas the group receiving id injections of tumor cells alone died with growing tumors and metastases in 52–65 days.

Sixty days later, each group of animals surviving in the above experiments were separated into subgroups of 5 animals each. Groups of 5 of similar ages and previously unsensitized rats served as controls. Five controls and 5 from each surviving group then received sc rejections of 5×10⁶ 13762 cells. Similarly, a group of controls and a group of surviving animals (5/group) received 5×10⁶ 13762 cells. Groups of rats exhibiting C. parvum-mediated rejection by means of id or sc injection of tumor cells admixed with killed C. parvum were protected and did not develop neoplasms after sc injections of the same numbers of living 13762 cells (table 1). Animals showing tumor cell rejection after being inoculated with cells admixed with BCG also had protection when given sc rejections of 10⁶ 13762 cells, but only 2 of 5 rats receiving the higher amount (sc rejections) of 13762 cells were protected. The remaining 3 rats died with slow-growing tumors and metastases. Unsensitized rats given 10⁶ and 5×10⁶ 13762 cells sc died predictably with growing tumors and metastases. These experiments were repeated with similar results (1 of 5 rats survived after rejections of 5×10⁶ tumor cells after inoculation of tumor cells mixed with BCG).

Subsequently, 3 of the rats (8/group) received one of the following id: 1) 5×10⁶ 13762 cells; 2) 5×10⁶ 13762 cells admixed with 1 mg killed C. parvum; or 3) 5×10⁶ 13762 cells admixed with BCG (10⁵ organisms). Similarly, 3 other groups of rats received sc injections of the inocula described above. The animals were killed at 10 days, and the tumors were excised and placed in 10% formalin in Hanks’ buffer for 24 hours. Microscopic evaluation of histologic sections (Giemsa stain) from the controls revealed an absence of cellular infiltrate. Intradermal growing tumors formed from inoculations of BCG or C. parvum had nonspecific macrophage infiltrates, but no lymphocytes were observed. We found that the subcutaneously growing tumors formed from inoculations of tumor cells and C. parvum had an organized infiltrate of macrophages surrounding each tumor follicle, whereas those formed from sc inoculation of cells and BCG revealed only a scanty, unorganized infiltrate.

We performed the following experiment to evaluate the nature of tumor rejection: Ten groups (10 animals/group) of previously unsensitized rats received id injections of one of the following: 1) 5×10⁴ 13762 cells mixed with 10⁵ BCG organisms (groups 1 and 2); 2) 10⁷ DMB 14 cells mixed with 10⁵ BCG organisms (groups 3 and 4); 3) 5×10⁴ 13762 cells mixed with 1 mg killed C. parvum (groups 5 and 6); 4) 10⁷ DMB 14 cells mixed with 1 mg killed C. parvum (groups 7 and 8); 5) 5×10⁴ 13762 cells; or 6) 10⁷ DMB 14 cells. All the animals in the groups receiving tumor cells admixed with either BCG or C. parvum formed tumors at the site of injection that were later rejected as described previously; rats given tumor cells alone died with growing tumors and metastases (table 2).

Three months after the start of the above experiment, each of the surviving groups was separated into 2 subgroups of 5 animals each. Four groups (5 animals/group) of previously unsensitized rats of similar ages served as controls. After rejection of tumors formed from inocula of cells admixed with BCG or C. parvum, groups of rats

---

**Table 1.** Protection in F344 rats given 13762 tumor cells plus living BCG and killed C. parvum id as compared with sc transplantation of tumor cells plus killed C. parvum.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial Route</th>
<th>Addition</th>
<th>Second Route</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>id 5×10⁶</td>
<td>1 mg killed C. parvum</td>
<td>sc 5×10⁶</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>id 5×10⁶</td>
<td>10⁷ living BCG</td>
<td>sc 5×10⁶</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>sc 5×10⁶</td>
<td>1 mg killed C. parvum</td>
<td>sc 5×10⁶</td>
<td>100</td>
</tr>
</tbody>
</table>

* Results following subcutaneous challenge injections of two different 13762 tumor-cell doses.
The following experiment was performed in order to observe the effect of BCG and *C. parvum* (injected at sites remote from the growing tumors) on recipients of tumor cell grafts.

Seventy (7 groups, 10/group) previously unsensitized rats received id inoculation of one of the following: 1) $5 \times 10^7$ 13762 cells alone; 2) $5 \times 10^6$ 13762 cells admixed with 1.0 mg *C. parvum*; 3) $5 \times 10^6$ 13762 cells followed by weekly ip injections of *C. parvum*; 4) $5 \times 10^6$ 13762 cells followed by weekly ip injections of BCG (10⁶ organisms); 5) $5 \times 10^6$ 13762 cells admixed with BCG (10⁶ organisms); 6) $5 \times 10^6$ 13762 cells followed by weekly id injection of living BCG (10⁶ organisms 1 cm away and around the injection site and growing tumor); and 7) $5 \times 10^6$ 13762 cells followed by weekly injections of killed *C. parvum* administered id 1 cm away and around the injection site and growing tumor. All injections of BCG or *C. parvum* were commenced a week after inoculation of tumor cells and were continued weekly until death.

The results illustrated in text-figure 2 revealed that tumor growth in all animals receiving tumor cells admixed with BCG or *C. parvum* was as expected for approximately 12 days, then the tumors disappeared. In the 10 rats given id inoculations of tumor cells followed by weekly id injections of *C. parvum* 1 cm away and around the growing tumor, lesions grew in 3 and then were rejected. The remaining 7 exhibited suppressed rates of tumor growth and prolonged survival (range, 108–120 days). Suppressed tumor growth and prolonged survival were also observed in all animals receiving weekly ip injections of *C. parvum* after id grafts of tumor cells (range, 68–82 days). All rats given id grafts first and then id injections of BCG 1 cm away and around the growing tumor died (range, 50–60 days) with growing tumors and metastases; their survival rates were comparable to the controls (range, 48–60 days). On the other hand, compared with controls the growth rates of tumors in rats receiving id inoculations of tumor cells before weekly ip injections of BCG were accelerated and early death ensued (range, 42–56 days). Gross examination by autopsy revealed that animals treated with ip doses of killed *C. parvum* had considerably fewer peritoneal and pulmonary metastases, whereas the rats treated with BCG had more metastatic nodules when compared with their respective controls. Similar results

![Text-figure 2](https://academic.oup.com/jnci/article-abstract/56/5/985/948424/Experimental-Cancer-Immunotherapy-Comparison-of)

by weekly ip injections of *C. parvum*; 4) $5 \times 10^6$ 13762 cells followed by weekly ip injections of BCG (10⁶ organisms); 5) $5 \times 10^6$ 13762 cells admixed with BCG (10⁶ organisms); 6) $5 \times 10^6$ 13762 cells followed by weekly id injection of living BCG (10⁶ organisms 1 cm away and around the injection site and growing tumor); and 7) $5 \times 10^6$ 13762 cells followed by weekly injections of killed *C. parvum* administered id 1 cm away and around the injection site and growing tumor. All injections of BCG or *C. parvum* were commenced a week after inoculation of tumor cells and were continued weekly until death.

The results illustrated in text-figure 2 revealed that tumor growth in all animals receiving tumor cells admixed with BCG or *C. parvum* was as expected for approximately 12 days, then the tumors disappeared. In the 10 rats given id inoculations of tumor cells followed by weekly id injections of *C. parvum* 1 cm away and around the growing tumor, lesions grew in 3 and then were rejected. The remaining 7 exhibited suppressed rates of tumor growth and prolonged survival (range, 108–120 days). Suppressed tumor growth and prolonged survival were also observed in all animals receiving weekly ip injections of *C. parvum* after id grafts of tumor cells (range, 68–82 days). All rats given id grafts first and then id injections of BCG 1 cm away and around the growing tumor died (range, 50–60 days) with growing tumors and metastases; their survival rates were comparable to the controls (range, 48–60 days). On the other hand, compared with controls the growth rates of tumors in rats receiving id inoculations of tumor cells before weekly ip injections of BCG were accelerated and early death ensued (range, 42–56 days). Gross examination by autopsy revealed that animals treated with ip doses of killed *C. parvum* had considerably fewer peritoneal and pulmonary metastases, whereas the rats treated with BCG had more metastatic nodules when compared with their respective controls. Similar results

![Text-figure 2](https://academic.oup.com/jnci/article-abstract/56/5/985/948424/Experimental-Cancer-Immunotherapy-Comparison-of)

by weekly ip injections of *C. parvum*; 4) $5 \times 10^6$ 13762 cells followed by weekly ip injections of BCG (10⁶ organisms); 5) $5 \times 10^6$ 13762 cells admixed with BCG (10⁶ organisms); 6) $5 \times 10^6$ 13762 cells followed by weekly id injection of living BCG (10⁶ organisms 1 cm away and around the injection site and growing tumor); and 7) $5 \times 10^6$ 13762 cells followed by weekly injections of killed *C. parvum* administered id 1 cm away and around the injection site and growing tumor. All injections of BCG or *C. parvum* were commenced a week after inoculation of tumor cells and were continued weekly until death.

The results illustrated in text-figure 2 revealed that tumor growth in all animals receiving tumor cells admixed with BCG or *C. parvum* was as expected for approximately 12 days, then the tumors disappeared. In the 10 rats given id inoculations of tumor cells followed by weekly id injections of *C. parvum* 1 cm away and around the growing tumor, lesions grew in 3 and then were rejected. The remaining 7 exhibited suppressed rates of tumor growth and prolonged survival (range, 108–120 days). Suppressed tumor growth and prolonged survival were also observed in all animals receiving weekly ip injections of *C. parvum* after id grafts of tumor cells (range, 68–82 days). All rats given id grafts first and then id injections of BCG 1 cm away and around the growing tumor died (range, 50–60 days) with growing tumors and metastases; their survival rates were comparable to the controls (range, 48–60 days). On the other hand, compared with controls the growth rates of tumors in rats receiving id inoculations of tumor cells before weekly ip injections of BCG were accelerated and early death ensued (range, 42–56 days). Gross examination by autopsy revealed that animals treated with ip doses of killed *C. parvum* had considerably fewer peritoneal and pulmonary metastases, whereas the rats treated with BCG had more metastatic nodules when compared with their respective controls. Similar results

![Text-figure 2](https://academic.oup.com/jnci/article-abstract/56/5/985/948424/Experimental-Cancer-Immunotherapy-Comparison-of)
were observed when these experiments were repeated (5 of 10 rats receiving id grafts of 13762 tumor cells before a weekly id injection of killed C. parvum was administered 1 cm away and around the growing tumors and survived tumor-free).

**DISCUSSION**

We found that tumor growth was suppressed in F344 rats after id inoculation of syngeneic 13762 mammary adenocarcinoma cells admixed with either living BCG or killed C. parvum. Tumor cells admixed with killed C. parvum and injected sc also resulted in rejection of the tumors formed from these grafts by the recipients. Furthermore, groups of rats bearing id tumor cell grafts exhibited suppressed rates of tumor growth and prolonged survival after being given killed C. parvum either ip or id 1 cm away and around the growing tumors. However, in similar experiments when living BCG was substituted for therapy (i.e., sc injections of tumor cells admixed with BCG; id inoculation of tumor cells before ip injections of BCG; and id inoculation of tumor cells and BCG 1 cm away and around the growing tumor), there was a lack of protection since these recipients died early with growing tumors and metastases. Animals showing tumor rejection mediated by killed C. parvum or living BCG exhibited tumor-specific protection to the sensitive cell line. In addition, animals having C. parvum-mediated tumor rejection exhibited protection to larger doses of challenge tumor cell grafts than did those showing BCG-mediated tumor rejection.

The function of macrophages in tumor rejection associated with immunoprotection was implied, since microscopic examination of histologic sections of tumors from animals displaying C. parvum or BCG-mediated tumor rejection revealed a non-specific infiltration of mostly granulocytes and macrophages in intradermally growing tumor nodules. The tumors regressing from initial sc transplants of cells admixed with C. parvum revealed a specific infiltration of many macrophages, polymorphonuclear granulocytes, and some lymphocytes around the tumor follicles. Minimal non-specific infiltration of mostly granulocytes and macrophages was seen in similar sections of tumors arising in animals given sc grafts of tumor cells admixed with BCG.

Although many similarities exist between living BCG and killed C. parvum in experimental cancer immunotherapy, there are also notable differences. In previous studies, Zbar (8) reported that he had optimal results with BCG with the use of a) living BCG, b) close contact between tumor cells and BCG, c) id administration, and d) a host capable of expressing a cell-mediated immune response. If these requirements were not observed, administration of BCG resulted in acceleration of tumor growth (9). In addition, C. parvum vaccine was effective in the treatment of subcutaneously growing tumors and appeared to enhance host resistance toward tumors and metastases after administration at a site other than that of a growing tumor (5-7). It was also beneficial in mice immunocompromised by thymectomy and treatment with rabbit antiserum to mouse thymocytes (10). Furthermore, C. parvum was effective in the treatment of metastases in experimental animals (6, 7, 11-16).

Animals receiving administrations of live BCG or killed C. parvum reflected an intense stimulation of the reticuloendothelial system and phagocytosis (17-19), steps that are essential for the induction of tumor-specific immune responses (20-24). Both immunostimulants also stimulate recruitment of lymphocytes into lymphoid structures in experimental animals (25-27). In addition, they induce thymus-dependent and -independent lymphocyte activity (7, 20, 21, 28-35) and also primate surveillance mechanisms (36-39) effective in graft-versus-host reactions. These studies also revealed that killed C. parvum was a more potent immunostimulant than BCG. Lamensoud and co-workers (40) reported that injections of killed C. parvum and lymphoma cells in AKR mice resulted in 50 and 83% survival, respectively, in syngeneic AKR and AKR (CBA/T6)F, mice, which suggested that there was also augmentation of the allogeneic effect. Furthermore, unlike observations with BCG, killed C. parvum was effective in thymectomized or T-cell depleted animals (10, 41).

In some of our recent experiments, we compared the efficacy of intratumor administration of live BCG and killed C. parvum in groups of rats bearing tumors at either intradermal or subcutaneous sites. Preliminary results indicate that BCG was effective only in the treatment of intradermally growing tumors and had no effect on metastases. Intratumor administration of tumors growing in rats at the intradermal and subcutaneous sites resulted not only in rejection of the growing tumors, but the conferred response was effective (unlike BCG) in the rejection of potentially lethal metastases.

Remarkable progress has been made in the field of cancer therapy since the first reports of experimental immunotherapy with the use of living BCG and killed C. parvum (42-45). Although both immunopotentiating substances have been employed in clinical cancer immunotherapy (46, 48), additional information is essential from similar experiments to determine the efficacy of these vaccines.

**REFERENCES**

(6) ———: Lasting rejection of mammary adenocarcinoma cell tumors in DBA/2 mice with intratumor injection of killed Corynebacterium parvum. Cancer Res 34:341-344, 1974


(22)EVANS R, ALEXANDER P: Role of macrophages in tumour immunity. I. Cooperation between macrophages and lymphoid cells in syngeneic tumour immunity. Immunology 23:615-626, 1972


(38)BIZZI G, HOWARD JC, MOUTON D, et al: Modification of graft versus host reaction induced by the pretreatment of the host with M. tuberculosi and C. parvum. Transplantation 3:170-177, 1965


