**Bacillus Calmette-Guérin Cell Wall Immunotherapy of Intramuscular and Metastatic Morris Rat Hepatomas**

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**ABSTRACT**

Treatment with *Bacillus Calmette-Guérin* cell wall attached to oil droplets (BCGcw) has little or no effect upon the growth of antigenic transplantable Morris hepatoma tumors, either of tumors growing at the i.m. site of tumor inoculation or of metastatic lesions in the lungs. When tumors weighing approximately 1 g were given injections directly on Day 10, 2 of 20 animals demonstrated regression of tumor growth, whereas injections into larger tumors (greater than 2 g) on Day 14 had no effect upon tumor growth. We performed three types of experiments using tumor lines 7777 and 5123tc to evaluate the effect of BCGcw immunotherapy upon the growth of tumor metastases. In Experiment 1, tumor line 7777 i.m. masses were amputated at a time when lung metastases would develop, and BCGcw were administered i.v. after amputation of the transplant site. Five of 27 (18%) of the animals treated with surgery only, and 9 of 57 (16%) of BCGcw-treated animals, survived free of metastases. With the use of the serum concentration of a-fetoprotein to follow the growth of metastatic lesions that arise following the i.t. injection of BCGcw, and subsequent tumor-specific immunity is sometimes established following BCGcw immunotherapy. In Experiment 2, "artificial" lung metastases were induced by the i.v. inoculation of either 1 x 10⁶ or 5 x 10⁴ tumor cells. Three to 6 hr later, animals were inoculated i.v. with 150 μg of BCGcw. This treatment had no effect upon the growth of metastases or upon the survival of animals. In Experiment 3, the inoculation of BCGcw into intrafootpad tumors prior to surgical excision also had no effect on metastatic tumor growth or survival. These studies suggest that BCGcw immunotherapy may not be effective upon the transplantable Morris hepatoma tumors studied.

**INTRODUCTION**

The growth of transplantable animal tumors may sometimes be restricted by general immunostimulation with BCG² (9, 10, 28). The i.l. injection of BCG and/or BCG agents into established tumors is capable of restricting tumor growth and of preventing the dissemination of metastases, and it may result in the cure of some animals (3, 8, 11, 21, 29). Recently, i.t. injection of BCG has been extended from peripheral sites to internal sites such as colon (12), pleura (17), peritoneum (17), and lungs (4, 5). The i.t. injection of BCG may establish a higher level of concomitant immunity than is seen with tumor growth alone. This concomitant immunity may, in part, be responsible for the inhibition of growth of metastatic lesions. The growth of a second tumor inoculum given to BCG-treated animals may be inhibited, but this effect may be overcome if a large number of tumor cells is inoculated (17). Complete local tumor regression may occur following the i.t. injection of BCG, and subsequent tumor-specific immunity is sometimes established (4). It is also possible that the treatment of established metastatic lesions that arise following the growth of an i.m. tumor mass may be successfully treated by the i.v. inoculation of BCGcw (2, 4–7). The present study, in which transplantable Morris hepatomas are used, addresses this possibility as well as the effect of BCGcw on artificial metastases induced by the i.v. inoculation of tumor cells and the effect of i.t. injection of BCGcw into established i.m. tumor masses.

The Morris hepatoma tumors studied in these experiments have tumor-specific transplantation antigens, but rechallenge resistance was only occasionally seen at 10 times the 100% lethal dose. These tumor cell lines may be similar in tumor antigenicity to spontaneous human tumors and may therefore provide models that reflect the effects of therapy on human cancers.

**MATERIALS AND METHODS**

**Animals.** Male ACI rats and male Buffalo rats (13 weeks old, 250 g) were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. They were housed in groups of 4 in stainless steel boxes with stainless steel grid tops and were supplied sawdust bedding, fresh water, and Purina laboratory rat chow *ad libitum*.

**Tumors.** All of the transplantable hepatomas described in this paper were originally obtained from Dr. H. Morris, Howard University, Washington, D. C. Tumor line 7777 is a poorly differentiated hepatocellular carcinoma induced after the dietary administration of N-2-fluorenylphthalimide.

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³ The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; i.l., intralesionally; i.t., intratumorally; BCGcw, *Bacillus Calmette-Guérin* cell walls attached to oil droplets; i.f., intrafootpad; AFP, α-fetoprotein; i.a., intrarterially; i.n., intranodally; i.d., intradermally.

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acid in a Buffalo rat. It was received from Dr. Morris in the 85th transplant generation, and transplant generations 95 to 126 were used in this study.

Tumor line 3924a is a poorly differentiated hepatocellular carcinoma induced after the dietary administration of N-2-fluorenylacetamide in an ACI rat. It was received from Dr. Morris in the 299th transplant generation, and transplant generations 303 to 310 were used in these studies.

Tumor line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced after the dietary administration of N-2-fluorenylphthalamic acid. The tumor was received from Dr. Morris in the 106th transplant generation. In this study transplant generations 115 to 125 were used.

**Tumor Cell Preparation.** Tumor cell preparations were made by mild trypsinization of small chunks of viable tumor tissue obtained from i.m. tumor as previously described (26). Cell viability was assessed with 0.1% trypan blue, as previously described, and all cell preparations contained 80 to 90% viable tumor cells (26).

**Preparation of BCGcw and Admixture with Tumor Cells.** The mycobacterial cell walls were obtained from Dr. Edgar Ribi, Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Mont., and were prepared in a Drakeol Tween 80-0.9% NaCl solution as previously described (15). The dosage of BCGcw used was 150 μg in a volume of 0.1 ml. The BCGcw were inoculated i.v. or i.t. Tumors located i.f. or i.m. were inoculated in 3 different locations.

**Amputation Procedure.** Tumor masses were excised from i.m. sites by the use of routine surgical procedures. Animals were anesthetized by the i.p. injection of pentobarbital (40 mg/kg). The surgical field was prepared in a routine antiseptic fashion, the skin incision was made, and the location of the tumor was noted to avoid excision through the tumor tissue. Proximal venous and arterial vessels were ligated, the tumor and adjacent soft tissue margin were excised, and the femur was amputated. The wound was closed with 4-0 silk suture, and autoclips were used to close the skin incision.

**Bleeding and Determination of AFP.** Blood samples were obtained by tail bleedings, and the serum AFP concentrations were determined by radioimmunoassay (18).

**Quantitation of Metastases.** At the completion of the experiments, animals were sacrificed by cervical dislocation, and complete postmortem examinations were performed. Tumor present in lungs and lymph nodes was quantitated by gross inspection. The presence and absence of metastatic disease was also determined microscopically. When i.m. tumor weights were determined, a gross dissection of the tumor away from the soft tissue was performed, and a tumor mass was weighed on a Mettler P1210 balance.

**Experimental Design.** The designs of the first 3 amputation experiments were basically similar in that animals were inoculated i.m. with tumor; after a given period of time the tumor was amputated, and the effect of BCGcw immunotherapy upon metastatic tumor growth was evaluated. In Experiment 1, animals were given injections with BCGcw, (150 μg i.v.) either 5 days before amputation, on the day of amputation, or 10 days after amputation. The average tumor mass on the day of amputation was 4.8 g. In Experiment 2, 1 group of animals received BCGcw i.v. on the day of amputation and another group received 2 doses of BCGcw (1 on the day of amputation and the other 8 days after amputation). The average tumor mass was 1.7 g. In Experiment 3, the dosage of BCGcw was varied, with 3 separate groups receiving 75, 150, and 300 μg, respectively. All injections were done on the day of amputation. The average tumor mass was 5.6 g. Animals given injections prior to amputation were selected at random. Animals given injections on the day of amputation were randomized into control and experimental groups on the basis of the weights of their excised tumors.

**Design of "Artificial Metastases" and Footpad Experiments.** Artificial metastases were caused by injecting tumor cells i.v., and BCGcw i.v. therapy was initiated 3 to 6 hr after tumor cell inoculation. In experiments dealing with footpad tumors, the footpad tumor mass was infiltrated with BCGcw 7 days prior to amputation of the tumor mass.

**Design of i.m. Tumor Experiments.** Animals were inoculated with 1 x 10⁵ tumor cells i.m. and 10 days later received BCGcw immunotherapy. In Experiment 1, there were 2 groups, a control group and the treated group. In Experiment 2, there were 6 groups. On Day 10, Group 1 was given i.t. injections of 150 μg of BCGcw; Group 2 was given injections of the same volume of diluent, and Group 3 was sacrificed to determine the average tumor mass on the day of therapy. On Day 14, when relatively large i.m. tumors were present, the above procedures were repeated with Groups 4, 5, and 6, respectively. In all experiments the animals were followed until they either died from tumor or were judged to be long-term survivors (6 to 7 months).

**RESULTS**

The results of Experiment 1 are summarized in Table 1. None of the 5 control animals survived postamputation. Three of 13 of the animals treated with the i.v. inoculation of 150 μg of BCGcw were long-term survivors. Of these survivors, 2 were from the 5 animals given injections on the day of amputation, and 1 was from the 4 animals given injections 10 days after amputation; none of the 4 animals given injections before amputation was a long-term survivor. All of the 10 experimental animals that died had extensive metastases to the periarterial lymph nodes and lungs. All animals were bled weekly, and their serum concentration of AFP in the control group was compared with the serum AFP concentrations of the 3 animals that were survivors following BCGcw treatment (Chart 1).

In the amputated control animals and in all of the experimental animals that died of metastatic tumor, there was an initial fall in AFP serum concentration followed by a marked increase in the AFP serum concentration that was associated with metastatic tumor regrowth. The AFP curves for the experimental animals with tumor (not shown) were similar to and indeed overlapped those of the control group shown in Chart 1.

All of the survivors had a fall in the serum concentration of AFP after amputation, followed by a transient elevation with return to normal levels. This suggested the occurrence of metastatic tumor that resolved. This transient elevation and subsequent fall did not occur in any other treated
animals. All 3 of the long-term survivors were rechallenged i.m. in their remaining hind legs with $2 \times 10^6$ tumor cells. One of the 3 survivors resisted rechallenge.

That the elevation of AFP in the treated animals was caused by a granulomatous response to the i.v. inoculation of BCGcw was excluded by the observation that normal animals inoculated i.v. with 150 $\mu$g of BCGcw had no elevation in their serum AFP concentrations.

The above experiment was repeated. In addition to a group of animals that received BCGcw the day of amputation, there was also a group that received 2 doses of BCGcw. The results of Experiment 2 are summarized in Table 1. Only 4 of 12 (33%) of the control animals treated with amputation survived free of metastases. One of 11 (9%) in Group 2 and 3 of 12 (25%) in Group 3 survived free of metastatic disease. All animals that died had extensive involvement of abdominal lymph nodes and lungs with metastatic tumor. There were fewer survivors in the BCGcw-treated groups (4 of 23, or 17%) than in the control group (4 of 12, or 33%).

The study was repeated, except the amount of BCGcw inoculated i.v. on the day of amputation was varied. The results are summarized in Table 1, Experiment 3. The survival rate was 1 of 10 (10%) in the control animals treated only by amputation of the i.m. tumor mass, and 2 of 21 (9.5%) in the BCGcw-treated animals. The 2 survivors were in the group treated with 75 $\mu$g of BCGcw i.v. The AFP serum concentrations were similar in both Experiments 2 and 3 in that all animals that had recurrent tumor had elevated serum AFP concentrations and all "cured" animals (experimental and control) did not have significant elevations of AFP after excision. This implies that they did not have recurrent metastatic tumor.

Although the above 3 experiments are not strictly comparable because they were done at different times with different tumor transplant generations, 5 of 27 (18%) of the amputated control animals were long-term survivors and 9 of 57 (16%) of the amputated and BCGcw-treated animals were long-term survivors. BCGcw i.v. immunotherapy as applied to this animal model was totally ineffective. The effect of inoculating BCGcw i.v. upon tumor growth in the lungs was tested. Animals were inoculated i.v. with either 1 $\times 10^6$ or $5 \times 10^6$ tumor cells from Tumors 7777 and 5123tc. Treated animals were inoculated i.v. with 150 $\mu$g of BCGcw approximately 3 to 6 hrs following the inoculation of tumor cells. No effect on survival, on number of lung metastases, or on AFP serum concentrations was observed.

We have shown that an i.f. inoculum of tumor cells and BCGcw was associated with a decrease in the development of lung metastases even when i.f. tumors grew. An experiment was undertaken to determine whether BCGcw inoculated into footpad tumors 7 days prior to surgical resection of the tumor would have any effect upon the growth of metastases and the overall survival. Forty animals were inoculated in the footpad with $3 \times 10^6$ line 7777 tumor cells and were treated by i.t. injection of BCGcw prior to amputation. The animals were randomized into 3 groups when footpad tumors were approximately 1.5 to 2.0 g in size; 5 animals were sacrificed at time of amputation (Group 1). Eighteen animals were inoculated with 150 $\mu$g of BCGcw i.t. 7 days prior to amputation (Group 2), and 16 animals were the un.injected controls (Group 3). Autopsy examination of Group 1 showed that, at the time of amputation, metastases were grossly visible in popliteal and periaortic lymph nodes and in the lungs. There were no noticeable differences between Groups 2 and 3; 7 animals in Group 2 and 8 animals in Group 3 were dead by Day 65. The remaining animals from both groups had approximately the same amount of metastatic tumor when sacrificed on Day 65.
We wished to determine whether inoculation of BCGcw into an established tumor mass would have any effect upon tumor growth. For this study, we used tumor line 3924a, which produces AFP in quantities that are directly proportional to the amount of tumor present. In Experiment 1, animals were inoculated i.m. and then on Day 10, a time when all animals had palpable tumors, 9 of 14 animals were inoculated with BCGcw, and 5 uninoculated animals served as controls. From previous studies, we estimated that the i.m. tumor mass was approximately 1 g. The results are presented in Table 2. On Day 22, 1 of 9 animals inoculated with BCGcw did not have a tumor, whereas a palpable tumor was detectable previously. The AFP serum concentration of this animal also dropped dramatically (Chart 2). Three other animals also had transient decreases in their AFP serum concentrations (not shown). No significant differences in either AFP serum concentration or the final tumor mass on Day 25 were observed between the experimental and control animals. The 1 survivor resisted i.m. rechallenge with $2 \times 10^6$ tumor cells.

In Experiment 2 (Table 2), groups of animals were treated with the i.t. injection of BCGcw on Day 10 as well as on Day 14 when relatively large i.m. tumors were present. The results are summarized in Table 2. The average tumor mass in the sacrificed animals was 1.21 ± 0.59 (S.D.) on Day 10, and on Day 14 it was 2.01 ± 0.65. As with the other experiments, 1 animal of 8 inoculated with BCGcw on Day 10 was cured, whereas the i.m. injection of BCGcw into tumors on Day 14 had no detectable effect upon tumor growth.

**DISCUSSION**

The majority of animal and human studies have established 3 general criteria that must be fulfilled in order for BCG or BCG-related agents to be effective in immunotherapy of tumors (9). These criteria are: (a) the tumor burden must be small; (b) there must be close contact between the tumor and the BCG; and (c) the tumor-bearing host must be able to mount an immune response to BCG and perhaps to the tumor antigen. Our studies on transplantable Morris hepatomas add additional support to these criteria.

Two major routes of inoculation with BCG have been used, namely, i.l. and i.v. Therapy i.l. may be followed by surgical removal within a short period of time. The i.l. injection of BCG is given with the hope that metastatic tumor growth may be inhibited. BCG may be inoculated i.v. admixed with tumor cells to prevent artificial metastases. Injections have also been given i.v. after surgical removal of a transplanted tumor and in studies done by others to cause a decrease in the incidence of tumor metastases even when tumor growth occurred locally. However, in this study little or no effect was noted upon survival or upon the incidence of lung metastases of this hepatoma when 150

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**Table 2**

<table>
<thead>
<tr>
<th>No. with tumor/total no.</th>
<th>Av. tumor mass when experiment was terminated*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Group 1. 150 µg BCGcw</td>
<td>8/9</td>
</tr>
<tr>
<td>inoculated into the tumor on Day 10</td>
<td>28.1</td>
</tr>
<tr>
<td>Group 2. Uninjected controls</td>
<td>5/5</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Group 1. 150 µg BCGcw</td>
<td>7/8</td>
</tr>
<tr>
<td>inoculated into tumor on Day 10</td>
<td>30.8</td>
</tr>
<tr>
<td>Group 2. Injected with diluent (Tween-0.9% NaCl solution) on Day 10</td>
<td>35.2</td>
</tr>
<tr>
<td>Group 3. Sacrificed on Day 10</td>
<td>Av. tumor mass 1.21 g</td>
</tr>
<tr>
<td>Group 4. 150 µg BCGcw</td>
<td>6/6</td>
</tr>
<tr>
<td>inoculated into tumor on Day 14</td>
<td>33.1</td>
</tr>
<tr>
<td>Group 5. Injection with tumor diluent (Tween-0.9% NaCl solution) on Day 14</td>
<td>35.3</td>
</tr>
<tr>
<td>Group 6. Sacrificed on Day 14</td>
<td>Av. tumor mass 2.01 g</td>
</tr>
</tbody>
</table>

* All experiments were terminated on Day 25.
μg of BCGcw were inoculated into large tumor masses in the footpad (1.5 to 2.0 g) prior to surgical excision. This therapeutic failure may have been due to the presence of a large amount of metastatic tumor at the time of BCGcw injection.

Studies to evaluate the efficacy of i.t. injection of BCG have generally shown that better results are obtained with small tumors (24, 27). BCG induced regression in approximately 60% of guinea pigs with 1-cm i.d. hepatoma transplants weighing 95 mg but in less than 20% of 1.5-cm tumors weighing 425 mg (27). The efficacy of BCGcw therapy may be site dependent. Littman et al. (14) reported that i.m. tumors growing in guinea pigs were resistant to the beneficial effect of i.l. injection of BCG, in contrast to the susceptibility of the same tumors growing i.d. One study with i.t. injections of heat-killed, formalin-fixed corynebacteria was successful in completely eradicating tumors that weighed approximately 2 g (1.6 cm) (13). In our experiments, when large (1-g) tumors were infiltrated with BCGcw, there was complete regression of 2 of 20 i.m. tumors. There was no effect when very large tumors (greater than 2 g) were treated. Studies in humans have shown that on occasion s.c. melanoma nodules will undergo regression following BCG inoculation (16).

I.v. Studies. Inoculation of BCG i.v. has also been shown to decrease the extent of metastatic tumor growth in some instances. When BCG or BCGcw are admixed with tumor cells and inoculated i.v., there may be a decrease in the incidence of artificial metastases (2, 5). In rats, the BCG inoculum can be given 6 or 7 days after tumor inoculations and still cause a marked decrease in the incidence of pulmonary tumors (2). The growth of pulmonary tumor metastases is more affected with highly antigenic tumor cell lines than with weakly antigenic lines (12). In the present study, the growth of artificial lung metastases was not affected by an inoculum of BCGcw 3 to 6 h after the i.v. inoculation of cells of Morris hepatoma lines 7777 and 5123tc.

Injection of BCG i.v. may also prevent metastases from a transplantation site. A prolonged survival and a significant reduction in the number of pulmonary metastases were noted when a spontaneously metastasizing rat epithelioma was excised 10 days after s.c. transfer, and the rats were then treated with an i.v. inoculation of BCG (2, 4). Our first amputation plus i.v. BCGcw therapy experiment seemed to confirm this observation. Three of 13 BCGcw-treated animals did not die of metastatic disease, whereas all of the untreated controls and therapeutic failures died with lung metastases. By following the serum concentrations of AFP, a protein produced by the tumor (19, 20), we found that there was a very strong suggestion that there was resolution of residual metastatic disease. One of the 3 survivors resisted tumor rechallenge, thereby demonstrating the presence of tumor immunity. Two subsequent experiments did not demonstrate any immunotherapeutic effect of the i.v. inoculation of BCGcw after the excision of the i.m. tumor mass, perhaps because of a change in the metastatic character of tumor line 7777. From transplant generation 85 to 129, a marked decrease in the incidence of lung metastases in association with the i.m. growth of the tumor was noted. This change in metastatic potential has remained constant in subsequent transplant generations. Relatively few metastases were found in untreated animals, so that significant differences between control and BCGcw-treated animals were not demonstrable. The administration of larger amounts of BCGcw led to a reduction in effectiveness of treatment. This is suggested by the reduced number of survivors in animals given 2 injections of BCGcw (Experiment 2) and the dosage study (Experiment 5). While BCGcw is a general immunostimulant, in some situations it has been shown to cause tumor enhancement (25).

Inoculation of BCG i.v. may also affect the spread of metastases from primary tumors. Treatment with BCG i.v. may also influence the appearance of pulmonary metastases in rats that develop hepatic tumors after long-term feeding on a diet that contains 4-dimethylaminoazobenzene (2, 7). BCG was given after completion of 90 days of carcinogen feeding and had no effect upon the primary hepatic tumors or upon the overall survival. Azuma et al. (1), using methylcholanthrene and 4-nitroquinoline to induce lung cancer in rabbits, showed that BCG therapy instituted at the start of carcinogen exposure and given repetitively caused a decrease in tumor incidence.

In summary, in the present experiments BCGcw immunotherapy had little or no effect upon the growth of transplantable hepatomas in rats. There are several possible explanations for this failure: (a) immunotherapy was initiated in animals with i.m. and intrapulmonic tumors when the tumor burden was beyond the capabilities of BCGcw immunotherapy; (b) the tumor lines studied are only moderately antigenic in that rechallenge resistance is rarely seen with a dose of tumor cells 10 x the 100% lethal dose or greater. Successful immunotherapy has been more frequently associated with highly antigenic tumors; (c) the anatomical location of the tumors makes them less susceptible to the therapeutic effects of the BCG; (d) the appropriate delayed hypersensitivity response of rats is less efficient than in species such as the guinea pig, where successful BCG therapy has been more easily demonstrated. Although we could demonstrate a delayed hypersensitivity response to BCGcw in rats, it was subjectively much weaker than that seen in guinea pigs.

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