Toxicity of Intrapleural Bacillus Calmette-Guérin Treatment in Animals

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ABSTRACT

The toxicity of intrapleural Tice strain Bacillus Calmette-Guérin (BCG) infection was tested in hamsters. Doses above 10^6 colony-forming units caused significant systemic infection, which could be controlled with conventional antituberculosis therapy. Living BCG in the pleural space did not prevent the healing of bronchial or vascular closures after pulmonary resection. Prophylactic intrapleural BCG (10^6 colony-forming units) significantly reduced tumor growth in the lungs of mice following i.v. injection of 5 x 10^6 syngeneic sarcoma cells. These animal experiments suggest that intrapleural BCG may be administered in the pleural space after lung resection in limited doses if followed by a complementary course of antimicrobial therapy.

INTRODUCTION

A retrospective chart study of patients who underwent resection for lung cancer at Albany Medical Center Hospital demonstrated improved survival for patients in whom an i.pl. infection developed postoperatively (22). A similar phenomenon has been reported in patients with peritonitis after colon cancer (18). These clinical observations prompted us to undertake a series of animal experiments designed to determine whether the beneficial effect of this infection could be reproduced safely. Administration i.pl. of attenuated Mycobacterium tuberculosis bovis Tice strain BCG was chosen to mimic the empyema because of its use as a vaccine against human tuberculosis with negligible morbidity and mortality (13) and its demonstrated effectiveness in reducing progressive growth of certain animal cancers (4). Lessons learned from these experiments ultimately allowed us to introduce BCG into the pleural space of humans as a planned equivalent of postoperative empyema (14, 15). This paper reviews the animal experiments on which our clinical trial of i.pl. BCG immunotherapy is founded.

MATERIALS AND METHODS

Animals. Six- to 16-week-old male C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) were used in all tumor experiments. Seven-week-old male and female white Syrian hamsters and 18- to 20-kg beagle dogs were obtained from the New York State Department of Health, Griffin Laboratories, Guilderland, N. Y.

Experimental Tumor. A syngeneic methylcholanthrene-induced fibrosarcoma, MCA 43, was kindly supplied by Dr. Charles McKhann (University of Minnesota) and was maintained in serial passage by s.c. injection of minced tumor into the thigh of C3H/HeJ mice. Single-cell suspensions for i.v. injection of tumor were prepared by digestion of minced tumor with 2.5% trypsin (25). The final suspension was diluted in Roswell Park Memorial Institute Culture Medium 1640 with 1% penicillin and streptomycin (Grand Island Biological Co., Grand Island, N. Y.). Cell viability was estimated by trypan blue exclusion, and the volume of the suspensions was adjusted to provide 5.0 x 10^6 viable tumor cells in 0.2 ml.

Mycobacteria and Antituberculous Drug Therapy. Lipophilized Tice strain BCG was obtained from Dr. Ray Crispin (University of Illinois), reconstituted with sterile 0.9% NaCl solution, and administered immediately. Each animal received 10^6 CFU of BCG in 0.2 ml unless otherwise noted. Injections (i.pl. and intramedial) were performed with a 22-gauge blunt thoracentesis needle with an orifice 1 mm from its tip (Popper and Sons, New Hyde Park, N. Y.). Intramedial injections were introduced at the suprasternal notch and i.pl. injections in the seventh intercostal space. Test injections using India ink showed that this technique consistently resulted in satisfactory placement of the inoculum without injury to the thoracic structures and without ink diffusion into adjacent serious spaces. Injections (i.c.) were given into the skin overlying the abdomen. Injections (i.v.) were given in the lateral tail veins. Dogs received BCG i.pl. at the time of pulmonary resection. In all experiments, control animals received 0.9% NaCl solution in an equal volume and at the same site as the BCG injection given to test animals.

One-half mg isoniazid (E. R. Squibb and Sons, New York, N. Y.) and 4.0 mg streptomycin (Chas. Pfizer and Co., New York, N. Y.) were injected i.p. into the hamsters every other day starting 8 days after i.pl. BCG injection and continuing until the hamsters were sacrificed 5 weeks later.

Pathology. Granulomata and acid-fast bacilli were sought microscopically in specimens of right parietal pleura, right lung, hilar lymph node, spleen, and right lobe of the liver in the hamsters and on specimens of lungs, pleura, and heart in mice. All specimens were obtained by using the aseptic technique and were fixed in 10% buffered formalin, dehydrated, and embedded in Paraplast. Sections of each were stained with hematoxylin-eosin and with carbol fuchsin.

Cultures of the pleura and liver were prepared by digestion with India ink (26), and tumor foci were sized and counted with an automatic bacterial colony counter (Model 870; Art Systems Corp., Farmingdale, N. Y.) (10).
stein-Jensen media containing 1\% penicillin and incubated in 10\% CO\textsubscript{2}. The cultures were monitored for 8 weeks after sacrifice by carbol fuchs in staining of samples of the cultures at 2-week intervals. Culture and characterization of the organisms were kindly performed by A. Loder and Dr. H. Gruff of the Division of Laboratories and Research, New York State Department of Health.

Statistics. Hypotheses were tested by using the Wilcoxon signed-rank test (11) and Steel's many-one rank statistic (17). These tests represent the nonparametric analog of the independent sample Student t test.

RESULTS

Studies in Hamsters

We chose hamsters for our preliminary toxicity studies because these animals are vulnerable to BCG infections (6). Administration i.d. of 10\textsuperscript{6} viable units of BCG regularly produced delayed sensitivity to old tuberculin in Syrian hamsters.\textsuperscript{4} Groups of 8 hamsters were given i.pl. injections of 10\textsuperscript{5}, 10\textsuperscript{6}, or 10\textsuperscript{7} viable units Tice strain BCG. Eight days later, 4 hamsters in each group were treated with 0.5 mg isoniazid and 4.0 mg streptomycin i.p. The antibiotic treatment was repeated every other day until the termination of the experiment 5 weeks later. Control animals received BCG but no antibiotics.

A thick organizing pleural reaction, rich in macrophages, was seen in all BCG-treated hamsters and was more pronounced in those animals given no antibiotics. Splenomegaly was pronounced in animals given 10\textsuperscript{7} viable units of BCG and no antibiotics. Acid-fast bacilli and granulomata were seen in the spleen, liver, pleura, and lymph nodes of all animals given BCG. Their number was dose dependent and was decreased, although not eliminated, by antibiotic therapy. A single granuloma was found in the lung of one BCG-treated animal. Viable units of BCG (10\textsuperscript{6})(10 times the i.d. immunizing dose) consistently gave a strong pleural and hilar lymph node granulomatous reaction.

The BCG organisms used were sensitive in vitro to 0.2 \( \mu \)g of isoniazid and 2.0 \( \mu \)g of streptomycin per ml.\textsuperscript{5} Cultures of spleen, liver, and lung were positive in all of the 8 hamsters given 10\textsuperscript{7} viable units BCG i.pl. followed by 5 weeks of antituberculous chemotherapy. None of 16 animals given 10\textsuperscript{6} or 10\textsuperscript{7} viable units of BCG and antituberculous chemotherapy showed positive cultures 5 weeks after injection. In hamsters given no antibiotic therapy, all cultures were positive 5 weeks after administration of BCG regardless of the dose.

Studies in Dogs

In anticipation of the use of i.pl. BCG as adjunctive therapy in human lung cancer patients, we evaluated the effect of i.pl. BCG infection on the healing of bronchial and vascular closures following pulmonary resections in dogs. Forty-eight pulmonary resections were performed (24 lobectomies and 24 pneumonectomies). Thirty-two dogs received 10\textsuperscript{6} viable units of BCG i.pl. at the time of closure, and 16 animals underwent resection alone without receiving i.pl. BCG. Isoniazid was not administered. The dogs were examined with serial X-rays and blood studies for 12 weeks. Mild leukocytosis and transient elevation of the serum alkaline phosphatase level followed BCG administration. The serum glutamic oxaloacetic transaminase level was not significantly elevated. Postmortem examination at the time of sacrifice demonstrated satisfactory healing of the bronchial stump with no air leakage at 50 mm mercury pressure in all dogs. A thick pleural reaction was seen in the dogs given BCG. Visceral granulomata in the liver and spleen were seen in 3 of 32 dogs given BCG, but acid-fast bacilli could not be cultured from any organ.

Effect of i.pl. BCG on Pulmonary Growth of i.v.-injected Syngeneic Sarcoma Cells in Mice

Timing. i.v. injection of a single-cell suspension of 5 \( \times \) 10\textsuperscript{6} MCA43 cells consistently caused visible tumor growth in the lungs of male C3H/HeJ mice by 14 days.

We compared the efficacy of i.pl. BCG in reducing pulmonary tumor growth in mice when the BCG inoculum was administered 1 day after and 4, 7, 14, and 21 days before tumor injection. Mice were sacrificed 14 days after i.v. injection of tumor. Prophylaxis was reliably effective in this experimental tumor model. Therapy was not. There was a 35\% reduction in tumor growth when BCG was given 1 day after tumor administration in one experiment (\( p < 0.05 \)) and no reduction in 2 others. A 76\% reduction in tumor growth could regularly be achieved when BCG was given prophylactically 21 days before tumor administration (\( p < 0.005 \) (Table 1)). Pretreatment with BCG 14 days before i.v. injection of tumor cells caused a 61\% reduction in tumor growth (\( p < 0.005 \)). Since this reduction in growth was not significantly different from that seen with 21-day pretreatment and was regularly reproducible, 14-day pretreatment was used in subsequent experiments for testing other variables.

Dose. We tested the effect of varying the dose of the i.pl. BCG inoculum at 14 days and found that 10\textsuperscript{8} CFU BCG gave optimal inhibition of tumor growth (\( p < 0.001 \) (Table 2)). Lower doses were less effective, and high doses resulted in the formation of extensive consolidating granulomata in the lung which obscured but did not eliminate tumor growth. Administration of 10\textsuperscript{7} or 5 \( \times \) 10\textsuperscript{7} CFU BCG did not reduce tumor growth more effectively than did 10\textsuperscript{8} CFU but resulted in the overgrowth of stainable acid-fast bacilli in the lungs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>Avg. tumor count</th>
<th>% of control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>139 ± 26\textsuperscript{6}</td>
<td></td>
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<tr>
<td>BCG - 21 days</td>
<td>10</td>
<td>33 ± 5</td>
<td>24 &lt;0.005</td>
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<tr>
<td>BCG - 14 days</td>
<td>10</td>
<td>54 ± 11</td>
<td>39 &lt;0.005</td>
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<tr>
<td>BCG - 7 days</td>
<td>8</td>
<td>70 ± 17</td>
<td>50 &lt;0.05</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>477 ± 28</td>
<td>52 &lt;0.015</td>
<td></td>
</tr>
<tr>
<td>BCG - 7 days</td>
<td>5</td>
<td>250 ± 60</td>
<td>58 &lt;0.002</td>
<td></td>
</tr>
<tr>
<td>BCG - 4 days</td>
<td>7</td>
<td>276 ± 34</td>
<td>58 &lt;0.002</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{6} The mice were sacrificed 2 weeks after i.v. injection of tumor cells. Lung tumors were sized and counted with an automatic bacterial colony counter (10).

\textsuperscript{5} The average tumor count of the test group divided by the average tumor count of the 0.9\% NaCl solution control.

\textsuperscript{6} Mean ± S.E.
Routes of Administration. Several routes of administration of BCG proved effective. There was no statistical difference between the i.pl., i.p., i.c., and i.v. routes of administration, all of which proved effective in inhibiting the growth of i.v.-injected tumor cells colonizing the lung (p < 0.001). In a separate experiment, the intramediastinal route was not as effective as the i.pl. route in inhibiting lung tumor growth [p < 0.15, not statistically significant (Table 3)].

DISCUSSION

The clinical observation that patients who developed bacterial infections in the pleural space after lung cancer surgery survived longer than patients without this complication represents a provocative natural experiment in immunotherapy (22, 24). In some series, the effect of postoperative infection has been indifferent (7) or negative (5). A beneficial effect from infection may be secondary to specific activation of the regional immune defenses against bacterial antigens, with a resultant accumulation of activated macrophages capable of nonspecific destruction of residual tumor cells (1, 16). Alternately, the infection may potentiate the immune response in a manner which facilitates specific sensitization to tumor antigens coresident in the lymphatic field (21), or the effect may be mediated by a nonimmunological mechanism.

Physical proximity of the bacteria to the tumor treated has been shown to be important when BCG infection is deliberately introduced to reduce the progressive growth of syngeneic hepatomas in animal models (2, 3, 27). Our experiments with mice did not demonstrate this requirement, but Pimm (20) has been able to provide a laboratory demonstration of this phenomenon in rats given i.pl. BCG to suppress an i.v.-injected hepatoma. While i.pl. BCG was effective in reducing tumor growth in our experimental model, i.v., i.c., and i.p. administration were equally effective. The relationship between the size of the dose of BCG and its rapid dissemination throughout the mouse or the fact that we measured pulmonary tumor growth rather than survival may have obscured a regional requirement in our experimental system.

Administration of i.pl. BCG 4, 7, 14, and 21 days prior to i.v. tumor challenge reduced tumor growth in the lungs of mice. The effect was greatest with 14 and 21 day immunoprophylaxis. This is consistent with the observation of Mackaness et al. (12) that the maximal regional lymph node response occurs approximately 14 days after stimulation with 10^6 CFU BCG and remains elevated for 28 days.

The dose of BCG administered appears to be crucial in eliciting an immunoprophylactic or immunotherapeutic effect. High doses of BCG result in systemic infection even when the BCG is given in a limited area. Hamsters given 10^7 viable units of BCG i.p. quickly developed disseminated infection of the liver and spleen. Similarly, humans given large intralesional inocula of BCG have developed systemic BCG infections (23). These quantitative considerations cannot be overstressed when BCG is administered to human patients. An increase from 10^7 to 10^8 CFU BCG in our mouse experiments resulted in an overgrowth of BCG in the pleural space and lung. Smaller doses of BCG were not as effective as 10^6 CFU in suppressing tumor growth. Chee and Bodurtha (8) made similar observations in studies of the growth of a s.c. melanoma in mice prestimulated once a week for 6 weeks with varying doses of BCG. A 10-fold increase or decrease from the optimal dose of 4 x 10^6 viable BCG organisms caused a 150 to 200% increase in tumor growth in their hands (8).

The variation in the average tumor counts in control animals in the mouse experiments may be related to the requirement for passage of the tumor every 10 to 14 days and the variation inherent in the steps which lead to lung metastases from the i.v. injection of tumor cells. Fidler (9) has characterized some of the host-tumor cell interactions, which influence the growth of artificial lung metastases, and has shown how the antigenicity and resultant rate of tumor growth shifts in a syngeneic tumor system through time. This shift is due to a selection of subpopulations of tumor cells within the syngeneic tumor which differ in metastatic potential (9, 19). Despite the variation in tumor growth in the control animals, 10^6 CFU BCG administered i.pl. 14 days prior to tumor injection consistently brought about a significant reduction in tumor growth in several experiments.

Although there are important differences between experimental animal systems and clinical cancer in humans, we feel that certain clear inferences can be made from the animal studies reported here which can be generalized for application in humans. We have shown that living BCG is safe to use in the chest after lung resection and does not interfere with the healing process. Timely administration of conventional antituberculosis drug therapy will control potentially overwhelming BCG infections. The murine tumor model has demonstrated...
that BCG administered in the pleural space can suppress the growth of experimental tumors in the lung when the critical parameters of dose and timing of the BCG inoculation are properly adjusted. Other microorganisms such as Corynebacterium parvum, Corynebacterium granulosum, Listeria monocytogenes, Nocardia rubia, or Bordetella pertussis may produce effects similar to those induced by BCG; however, their safe use in humans is not as well documented historically.

In light of these observations, we have initiated a randomized trial of i.pl. BCG immunotherapy in humans. The early results of this trial suggest that the i.pl. administration of BCG, when given in proper dosage and followed by antituberculous chemotherapy, is safe and may be efficacious in reducing the progression or recurrence of resectable lung cancers (14, 15).

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REFERENCES


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Toxicity of Intrapleural *Bacillus Calmette-Guérin* Treatment in Animals
