

Suppression and Immunotherapy of the Guinea Pig Line 10 Hepatocarcinoma Mediated by Heat-killed Disrupted *Mycobacterium bovis* Strain *Bacillus Calmette-Guérin*¹

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ABSTRACT

Heat-killed *Mycobacterium bovis* strain *Bacillus Calmette-Guérin* cells were sonically disrupted, and their antitumor effects against the line 10 hepatocarcinoma in strain 2 guinea pigs were evaluated. When injected together with viable line 10 cells, there was complete suppression of tumor growth. Growth of tumor was also suppressed when line 10 cells were injected contralaterally at the same time as the vaccine mixture. Multiple intratumor injections of sufficient disrupted *M. bovis* strain *B. Calmette-Guérin* were therapeutically effective against 74% of 7-day-old tumors and against 40% of 14-day-old tumors. Surviving animals were usually resistant to subsequent rechallenges with line 10 cells but not to syngeneic L₂C leukemia cells. By means of a competitive radioimmunoassay, antigenic determinants were detected that were expressed by disrupted but not by intact bacteria.

INTRODUCTION

Viable BCG⁴ cells have been shown to be effective for treatment of the line 10 hepatocarcinoma in strain 2 guinea pigs when injected directly into existing tumors. Metastases in neighboring lymph nodes were also eradicated, and treated animals were resistant to subsequent challenges of line 10 cells (2, 17, 21). Subcellular components of BCG were similarly effective when administered together with oil droplets and trehalose dimycolate (18). Recently, it was shown that multiple intratumor injections of a variety of intact heat-killed bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, *Propionibacterium acnes*, and *Listeria monocytogenes* administered without oil droplets or trehalose dimycolate were also therapeutically effective (5, 12). Injections of heat-killed BCG, however, under the same and similar conditions were ineffective (3, 5, 17). BCG is known to possess a relatively impermeable lipid cell wall (16), and it is possible that components or antigens critical for their antitumor effects are inappropriately exposed on intact nonviable BCG.

In this study, heat-killed BCG cells were sonically disrupted

and then tested for their antitumor effects. It was found that completely disrupted nonviable BCG suppressed growth of line 10 tumors and caused regression of established lesions. Cured animals were resistant to subsequent challenges with line 10 cells.

MATERIALS AND METHODS

BCG. BCG (Glaxo) was obtained from the culture collection of this institution and grown as previously described (12, 13). The bacteria were killed by inspissation at 80° for 40 min and washed with sterile pyrogen-free water. Suspensions of the killed washed mycobacteria were sonically treated until by microscopic examination greater than 95% were disrupted. This took 60 to 90 min. This material is referred to as BCG sonicate. Portions of the sonically disrupted material were subjected to ultracentrifugation for 2 hr at 200,000 × g. The resulting supernatant fluid is referred to as BCG-S, and its characteristics have been described (1).

Animals. Male and female strain 2 guinea pigs weighing 350 g or more were obtained from the breeding colonies of the Frederick Cancer Research Center and from this institution. New Zealand White rabbits were used as a source of rabbit sera.

Tumors. The diethylnitrosamine-induced line 10 hepatocarcinoma of strain 2 guinea pigs was maintained in ascites form. Line 10 cells used in these experiments were of transplant generations 6 to 15. Injections of 10⁶ viable ascites line 10 cells intradermally into strain 2 guinea pigs invariably cause growing tumors and death of recipients within 60 to 90 days (17). L₂C leukemia cells (1a positive) were provided by Dr. A. Cooper, Tufts University School of Medicine, Boston, Mass. Injections of 3 × 10⁵ L₂C cells intradermally into normal animals result in fatal leukemias in 2 to 3 weeks; L₂C-immune animals resist this challenge (8).

Sera. Rabbits received inoculations of BCG sonicate in incomplete Freund's adjuvant as previously described, and their antisera are referred to as anti-BCG (14).

Measurement of Antibodies. BCG-S was labeled with ¹²⁵I as previously described (14). ¹²⁵I-labeled BCG-S was diluted so that the cpm and nitrogen concentrations in 0.1 ml were 10,000 to 15,000 and 0.01 μg nitrogen, respectively. The nitrogen content of unlabeled and radiolabeled antigens was determined by an automated micro-Kjeldahl method (7). The capacity of anti-BCG to bind radiolabeled antigens was tested by reacting dilutions of antisera with ¹²⁵I-labeled BCG-S (1:5). Dilutions of antisera were in BB (11). Radiolabeled antigen-antibody complexes were then precipitated with anti-rabbit IgG prepared in goats. Results are expressed as cpm in resulting precipitates. In inhibition experiments, 0.1 ml of antisera was

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⁴ The abbreviations used are: BCG, *Mycobacterium bovis* strain *Bacillus Calmette-Guérin*; BCG sonicate, sonically disrupted *B. Calmette-Guérin*; BCG-S, soluble preparation of *B. Calmette-Guérin* sonicate; anti-BCG, antisera in rabbits inoculated with *B. Calmette-Guérin* sonicate in incomplete Freund's adjuvant; BB, borate buffer.

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preincubated overnight with 0.1 ml of BB, BCG sonicate, or suspensions of intact heat-killed BCG. ¹²⁵I-labeled BCG-S was then added, and binding capacities of sera were determined as previously described (14). Percentage of inhibition was calculated as follows:

$$1 - \frac{\text{cpm in precipitates after preincubation with BCG or BCG sonicate}}{\text{cpm in precipitates after preincubation with buffer}} \times 100$$

RESULTS

Suppression of Growth of the Line 10 Hepatocarcinoma by BCG Sonicates. Strain 2 guinea pigs received injections intradermally into their left flanks with 10⁶ line 10 cells suspended with 220 μg of nitrogen of BCG sonicate in volumes of 0.3 ml. After the line 10 cells were mixed with the sonicate, viability by trypan blue dye exclusion was 90 to 95%. In most animals, there was slight induration at the injection sites which disappeared within 3 days. In all the animals tested, there was complete tumor suppression (Table 1). When rechallenged with 10⁶ line 10 cells 60 days after the beginning of the experiment, no tumors developed. In one experiment, BCG sonicate was injected into the left flanks, and 10⁶ line 10 cells were injected separately into the right flanks. Tumors grew on the right flanks, which indicated that close contact of the sonicate with tumor cells was required for suppression to occur. In another experiment, the vaccine effect of the BCG sonicate:line 10 cell mixture was studied. A group of animals received intradermal injections of the BCG tumor vaccine on one flank and line 10 cells alone on the opposite flank on the same day. In 4 of the 5 animals, there was no tumor growth on either side. When the 4 animals were rechallenged intradermally with line 10 cells alone about 60 days after the beginning of the experiment, all demonstrated delayed cutaneous hypersensitivity reactions to the line 10 cells, and 3 of the 4 did not develop tumors (Table 1).

Therapeutic Effect of Multiple Intralesional Injections of BCG Sonicate. Experiments were performed to test whether

the BCG sonicate could influence growth of existing line 10 tumors. Other studies in which similar protocols were used have shown that microscopic metastases within regional nodes are present within 6 days after the intradermal injection of 10⁶ cells (17, 21). Guinea pigs were given intradermal inoculations of 10⁶ line 10 cells, and on Day 7, when tumor diameters were approximately 10 mm, lesions were infiltrated with BCG sonicate in 0.3-ml volumes. Intralesional injections were given 3 times on Days 7, 14, and 21.

Following intralesional injections, the tumor papules slowly increased in size, the largest diameter noted being 20 mm. Small areas of ulceration appeared on the tumor surface, but in most instances after the third injection on Day 21, the papules gradually diminished in size, completely disappearing between Days 25 and 40. Of the 23 animals treated with multiple injections of 130 to 220 μg of nitrogen sonicate, there was complete regression in 17 (74%) (Table 2). In some cured animals, scar tissue remained at the tumor site. Sixty days after the onset of the experiments, 15 animals were rechallenged with line 10 cells alone. They all developed delayed cutaneous hypersensitivity reactions, and 13 (87%) were completely resistant to this challenge. Tumor regression was related to the amount of sonicate injected. Single intralesional injections of BCG sonicate (220 μg of nitrogen) into 7-day tumors resulted in eradication of a tumor in only 1 of 6 animals so tested. In another experiment, tumors were allowed to develop for 14 days. These lesions were then treated as above with 3 weekly injections of BCG sonicate. In 4, there was complete tumor regression, and 2 of these resisted a subsequent rechallenge with line 10 cells alone. Animals not completely resistant to rechallenge had prolonged latent periods before tumors were evident, and survival time was considerably increased compared to unprotected controls. Twelve of the survivors were then challenged with 3 × 10⁵ L₂C cells, and all developed fatal leukemias.

Comparison of the Capacity of Dead Intact BCG and of BCG Sonicates to Inhibit the Binding of ¹²⁵I-labeled BCG-S by Anti-BCG. Because disrupted BCG had *in vivo* antitumor effects and because dead intact BCG did not (3, 5, 17), experiments were carried out to see if differences in the antigenic expression of these 2 BCG preparations could be de-

Table 1

Suppression of line 10 hepatocarcinoma by sonicate of heat-killed BCG

Treatment	No. of animals tumor free/no. treated	Rechallenge ^a (No. of animals tumor free/no. challenged)
10 ⁶ Line 10 cells and BCG sonicate ^b	5/5 (100) ^c	4/4 (100)
10 ⁶ Line 10 cells and BCG sonicate in contralateral flanks ^d	0/4 (0)	
Line 10 cell:BCG sonicate vaccine and line 10 cells alone in contralateral flanks ^e	4/5 (80)	3/4 (75)
10 ⁶ Line 10 cells alone	0/5 (0)	

^a Animals surviving initial treatment were rechallenged intradermally with line 10 cells alone on the opposite flank after about 2 months and observed for at least 60 days.

^b Strain 2 guinea pigs were given inoculations intradermally with 10⁶ line 10 cells mixed with BCG sonicate (220 μg nitrogen).

^c Numbers in parentheses, percentage.

^d Animals received 10⁶ line 10 cells intradermally in the right flanks and BCG sonicate (220 μg nitrogen) in the left flanks on the same day.

^e Animals received 10⁶ line 10 cells intradermally in the left flanks. Vaccine consisting of 10⁶ line 10 cells and BCG sonicate (220 μg nitrogen) was injected intradermally into the right flanks on the same day.

Table 2

Therapeutic effect of multiple intralesional injections of sonicate of heat-killed BCG

Age of tumor treated	Dose of BCG sonicate (μg nitrogen)	No. of animals tumor free/no. treated	Rechallenge ^a (No. of animals tumor free/no. challenged)
7 days ^b	10	0/6 (0) ^c	
	20	2/6 (33)	2/2 (100)
	130	4/6 (67)	4/4 (100)
	220	13/17 (71)	9/11 (82)
	Total (130 or 220 μg nitrogen)	17/23 (74)	13/15 (87)
14 days ^d	220	4/10 (40)	1/4 (25)
Controls (untreated)		0/12 (0)	

^a Most animals surviving the treatment were rechallenged with 10⁶ line 10 cells alone after about 2 months and were observed for at least 60 days.

^b Animals were inoculated with 10⁶ line 10 cells intradermally and then intralesionally with the BCG sonicate on Days 7, 14, and 21.

^c Numbers in parentheses, percentage.

^d Intralesional injections of BCG sonicates were made on Days 14, 21, and 28.

tected. Sera from rabbits that had been immunized with the BCG sonicates were diluted 1:5, and 0.1-ml aliquots were incubated with BB, dilutions of suspensions of dead intact BCG, or BCG sonicates. The sonicates tested consisted of equivalent numbers of intact BCG. Concentrations of the sonicated and intact BCG in these experiments were adjusted so that the dry weights of each preparation were the same, 35 mg/ml. As shown in Chart 1, 0.5 ml of sonicate inhibited the reaction between ¹²⁵I-labeled BCG-S and anti-BCG by 64%, whereas an equivalent amount of intact BCG did so by only 34%. Smaller amounts of BCG or sonicate inhibited this reaction to a lesser degree, but disrupted BCG in the sonicates was always more effective.

DISCUSSION

The experiments described above demonstrated that sonically disrupted BCG could suppress growth of the line 10 tumor when injected together with viable tumor cells. In addition, when sufficient BCG sonicates were injected, there was complete tumor regression in 17 of 23 animals with existing 7-day-old tumors and in 4 of 10 animals with 14-day-old tumors. Using similar protocols, microscopic metastases have been demonstrated in neighboring lymph nodes within 6 days after injection of tumor cells (17, 21). Recovered animals were generally resistant to subsequent challenges with line 10 cells alone but not to the syngeneic L₂C tumor. Therapeutic effectiveness was almost identical with those reported following treatment of this tumor with viable BCG or with subcellular bacterial components administered with oil droplets and trehalose dimycolate (17, 18).

As previously reported, single or multiple intralesional injections of various numbers of intact heat-killed BCG do not affect tumor growth (3, 5, 17). In a competitive inhibition assay, there were differences in antigenic expression between intact and disrupted BCG preparations in that antigens expressed by disrupted BCG were not detected in intact killed BCG. The antitumor effects of disrupted BCG may thus have been due to antigenic determinants which are not optimally expressed *in vivo* on the surfaces of dead intact BCG. It is known that BCG shares antigens with the line 10 tumor (4, 6, 10, 14). Antigenic determinants exposed by the disruption of BCG, including those shared by BCG and line 10 cells, could have been important for tumor rejection and may have contributed to the antitumor effects of metabolically active viable BCG.

In vivo multiplication of BCG organisms is required for effec-

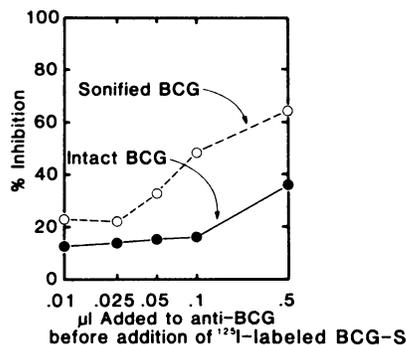


Chart 1. Inhibition of binding of anti-BCG to ¹²⁵I-labeled BCG-S by unlabeled intact and sonified BCG.

tive immunization against tuberculosis, and it has also been considered to be essential for the antitumor effects of BCG (9). However, antigenic stimulation by preparations of dead BCG may occur without the need for multiplication *in vivo* (2). This is particularly probable when, as in this study, disrupted BCG cells are administered by multiple injections or, as in other studies, when bacterial components are injected suspended in oil droplets resulting in a persistent source of antigenic stimulation (19).

The mechanism(s) by which BCG influences the growth of tumors and renders many animals specifically resistant to rechallenge is not known (2, 15, 20). Recently, small amounts of BCG-derived peptidic substances combined with oil and various adjuvants have been shown to cause regression of the line 10 tumor (19). Results of the present study raise the possibility that antigens expressed by metabolically active BCG and by disrupted BCG (but not by inappropriately exposed antigens in dead intact BCG) may be of significance.

Viable BCG is currently used for the treatment of a variety of tumors. If disrupted BCG is found to be similarly effective against other tumors, then it may merit consideration as a satisfactory substitute for viable BCG in order to obviate the disadvantages of immunotherapy with viable bacteria.

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