

## Effects of BCG, *Corynebacterium parvum*, and Methanol-Extraction Residue in the Reduction of Mortality from *Staphylococcus aureus* and *Candida albicans* Infections in Immunosuppressed Mice

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An immunosuppressed mouse model was devised to test the effects of immunopotentiators on the prevention of bacterial and fungal infections. The effects of BCG and *Corynebacterium* were tested against *Staphylococcus aureus* and *Candida albicans* infection. The effect of methanol-extraction residue (MER-BCG) was tested against *S. aureus* septicemia. CDF<sub>1</sub> mice were given various doses of BCG, 1.0 mg of *C. parvum*, or 0.5 mg of MER intraperitoneally at varying intervals before injection of an intravenous bacterial challenge. Four days before challenge, 300 mg of cyclophosphamide per ml was given intraperitoneally. BCG (10<sup>6</sup> colony-forming units) reduced mortality due to *S. aureus* at pretreatment intervals of 3, 7, 14, and 28 days. Isonicotinic acid hydrazide treatment eliminated the protective effect of the live BCG. *C. parvum* was as effective as BCG against *S. aureus* septicemia when given 3 days before infection, but lost most of its protective effect after that time. MER protected at doses as small as 0.25 mg when given 25 days prior to challenge. Both BCG and *C. parvum* exerted a protective effect against *Candida albicans* infection.

Animals infected with live mycobacteria, i.e., BCG, acquire resistance to a variety of heterologous pathogens, including *Staphylococcus aureus* (5), *Salmonella* (7, 17), and *Herpesvirus hominis* (10), as well as to tuberculous infection. Other immunostimulators, such as methanol-extraction residue (MER) of BCG (23-25) and *Corynebacterium parvum* (1, 3) agents currently used in cancer immunotherapy, have also been reported to induce similar resistance-enhancing effects. Cancer patients treated with immunosuppressive drugs often develop serious bacterial and fungal diseases. Immunotherapeutic efforts with BCG, MER, and *C. parvum* attempt to restore antitumor specific and non-specific immune functions to the host. Besides the potential antitumor benefits, it is possible that increased resistance to opportunistic bacterial and fungal diseases may be induced. In these investigations, several parameters of antibacterial and antifungal resistance have been studied in an immunosuppressed mouse model by using high doses of cyclophosphamide and

subsequent intravenous challenge with either *S. aureus* or *Candida albicans*.

### MATERIALS AND METHODS

**Animals.** CDF<sub>1</sub> male mice were kindly supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, through the Laboratory Supply Co., Indianapolis, Ind. The mice were housed in plastic filter-top cages and fed Purina mouse pellets and water *ad libitum*. All mice were at least 23 g.

**BCG vaccine.** The Brazil strain of *Mycobacterium bovis* was obtained from the Trudeau Mycobacteria Culture Collection, Saranac Lake, N.Y., prepared as previously described (18), and stored frozen at -70 C. The concentration of the stock vaccine in colony-forming units was determined by the method of Rosenthal et al. (16). Immediately before use, the vaccine was rapidly thawed in a 37 C water bath and diluted with phosphate-buffered 0.85% NaCl, pH 7.2, and injected intraperitoneally (i.p.). Controls received 0.1 ml of phosphate-buffered saline. Counts of viable BCG in the spleen were determined as previously described (18).

**Cyclophosphamide.** Cytoxan (Mead-Johnson, Evansville, Ind.) was freshly prepared for each experiment and administered in a dosage of 300 mg/kg, i.p. Isonicotinic acid hydrazide (Eastman Chemicals, Rochester, N.Y.) in a powder form was dissolved in

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drinking water at a concentration of 0.5 mg/ml. The bottles were covered with foil and changed daily.

**Bacteria.** *S. aureus* (strain Giorgio) was obtained from the American Type Culture Collection no. 13710, Rockville, Md. A clinical isolate of *C. albicans* was obtained from the Clinical Microbiology Service of the National Institutes of Health. The organisms were subcultured daily for 3 days in broth at 37 C and, to maintain virulence, passed twice through CDF<sub>1</sub> mouse lungs. They were then passed two times prior to inoculation on nutrient agar (*S. aureus*) or Sabouraud agar (*C. albicans*). Surface growth was harvested in skim milk, and the bacteria were freeze dried and stored in sealed glass vials at 20 C in aliquots of 0.2 ml. For each experiment, the freeze-dried bacteria were reconstituted with sterile distilled water and serially diluted with phosphate-buffered saline to the appropriate concentrations. All *S. aureus* inocula were counted at 24 h in duplicate on Tellurite glycine agar. *C. albicans* were serially counted on Sabouraud agar.

*C. parvum* and MER were kindly supplied by J. P. Davignon of the Cancer Therapy Evaluation Branch of the National Cancer Institute. *C. parvum*, Institute Merieux, Lyon, France (lot SO274, 2 mg/ml), was kept at 4 C and administered i.p. in a dose of 1.0 mg. MER was supplied in powder form (lot L674738-00G07, Merck Co., Rahway, N.J.), reconstituted in a steroid-suspending vehicle (hydroxypropyl cellulose, 0.3% solution) to a concentration of 1 mg/ml, and administered i.p.

**Statistics.** All means were arithmetic. The *P* values were based on Student's *t* test. A value of *P* > 0.05 was considered nonsignificant. Unless otherwise indicated, a *P* value will represent a comparison between the treatment group and its appropriate control.

**Experimental procedure.** Groups of mice were injected i.p. with either BCG, *C. parvum*, MER, or saline at various intervals before inoculation of the intravenous bacterial challenge on day 0. Cyclophosphamide was given in a dose of 300 mg/kg, 4 days before the challenge. The 50% lethal dose for each of the bacterial challenges was determined in cyclophosphamide-treated mice in a series of preliminary experiments. All groups were then monitored every other day for death. Experiments were terminated at 50 days, as there was little change in final outcome after this time. For statistical comparison, the arithmetic means of the individual days of death were calculated. This will be referred to as the mean day of death.

## RESULTS

**Effect of BCG in non-immunosuppressed mice.** Without cyclophosphamide, the 50% lethal dose for both organisms was 2 to 3 log<sub>10</sub> higher than with immunosuppression. To illustrate the protective effects of BCG, two groups of 25 mice were injected with either 0.1 ml of phosphate-buffered saline PBS or 10<sup>7</sup> colony-forming units of BCG 14 days before intravenous challenge with 2.5 × 10<sup>8</sup> *S. aureus*.

The mean day of death was increased from 15 to 23.2 days with the BCG pretreatment (Fig. 1).

**Effect of cyclophosphamide.** To measure the point of optimal immunosuppressive effect of the cyclophosphamide, a dose of 300 mg/kg was given i.p. to groups of mice. A decrease in the total leukocyte count reached its nadir by day 4 and gradually returned to normal by day 8 (Fig. 2). There was a decrease in the spleen weight, which returned to normal after 1 week. This high dose of cyclophosphamide resulted in weight loss and morbidity. There was a mortality of approximately 10%. All subsequent experiments reported here will be performed using cyclophosphamide immunosuppression.

**Effect of various intervals of immunostimulator pretreatment on survival from *S. aureus* septicemia.** BCG in doses of 10<sup>6</sup>, 10<sup>7</sup>, or 10<sup>8</sup> colony-forming units/0.1 ml or *C. parvum* in a dose of 1.0 mg/0.1 ml was given at intervals of 3, 7, 14, or 28 days prior to an intravenous challenge with *S. aureus*. The mean dose of *S. aureus* ± standard error was 1.68 ± 0.20 × 10<sup>5</sup>. Cyclophosphamide was given 4 days before the challenge. The results are illustrated in Fig. 3. The number of mice used in each experiment is indicated in parentheses. BCG at 10<sup>6</sup> colony-forming units reduced mortality at each of the pretreatment intervals (3, 7, 14, and 28 days). Smaller doses of BCG were not significantly more effective than the control group. *C. parvum* was as effective as 10<sup>6</sup> BCG when given 3 days before the challenge but lost most of its protective ability when administered at 7, 14, or 28 days before challenge. A similar experiment performed at day 56 (not shown in Fig. 3) showed continued protection by the BCG but no protection from the *C. parvum*.

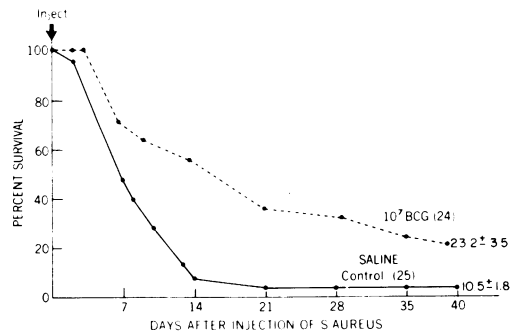


FIG. 1. Effect of BCG pretreatment on survival from *S. aureus* septicemia without cyclophosphamide immunosuppression. Groups of 25 mice were given either saline or 10<sup>7</sup> colony-forming units of BCG 14 days before intravenous challenge with *S. aureus*. The mean day of death is indicated for each group.

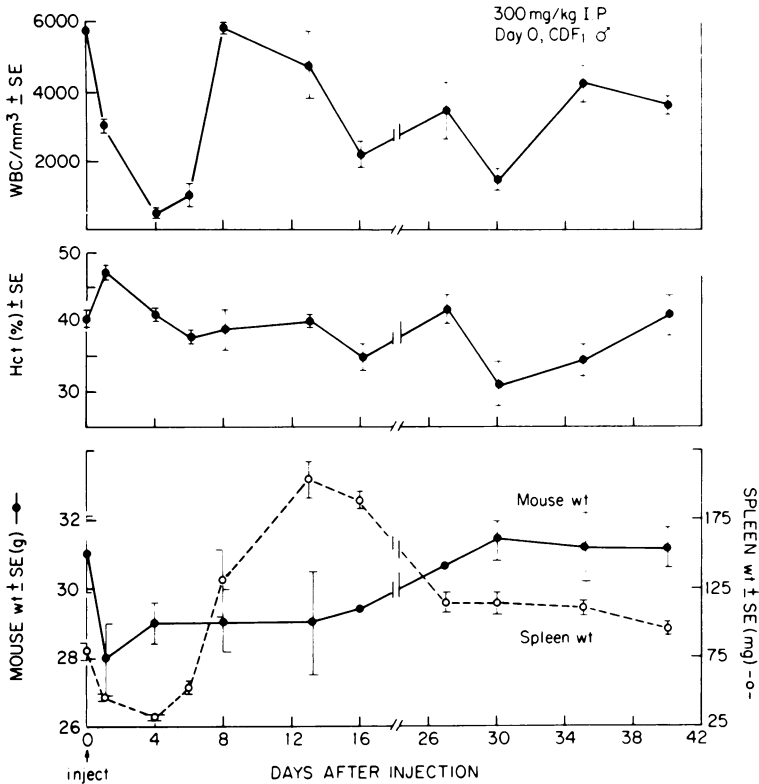


FIG. 2. Host response to cyclophosphamide. A group of CDF<sub>1</sub> mice was injected with 300 mg of cyclophosphamide per kg, i.p., day 0. Groups of four to five mice were sacrificed at various intervals, and leukocyte count (WBC), hematocrit (Hct), spleen weight, and body weight were determined. All values represent arithmetic mean ± standard error (SE).

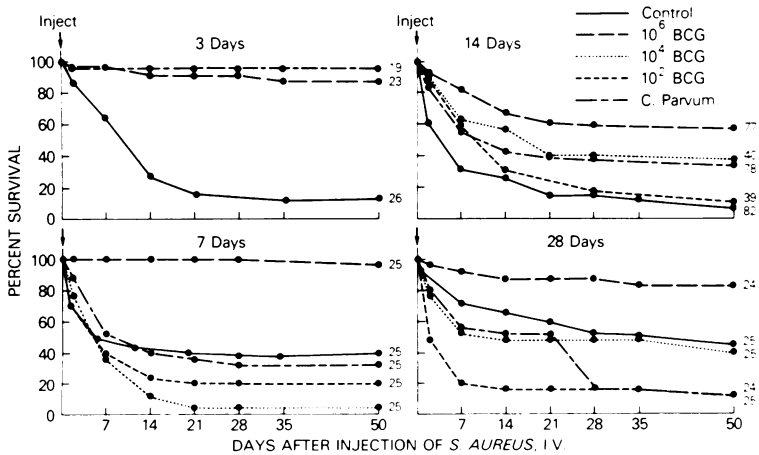


FIG. 3. Effect of various intervals of immunostimulator pretreatment on survival from *S. aureus* septiemia. See text for details. Numbers in parenthesis indicate the number of mice in each group.

**Effect of various doses of MER.** Groups of mice were pretreated with various doses of MER or 0.5 ml of the steroid-suspending vehicle, i.p., 14 days before challenge. All animals

were treated with cyclophosphamide as usual. Some morbidity, including ataxia, was observed with the 1.0-mg dose of MER. The mean day of death was increased with all dose levels,

whereas the survival was increased only with the 0.5- and 1.0-mg doses (Table 1).

**Effect of isoniazid treatment on the protective ability of BCG.** Isoniazid treatment started 1 day before BCG administration prevented the growth of viable BCG, as shown by culturing the spleen at day 14. No mycobacteria could be cultured at this time. Isonicotinic acid hydrazide treatment diminished the protective effect of BCG and lowered the survival rate to that of the group receiving isonicotinic acid hydrazide alone ( $P < 0.01$ ). Isonicotinic acid hydrazide treatment alone exerted a small but significant effect (Fig. 4).

**Effect of BCG and *C. parvum* on *C. albicans* septicemia.** Results of experiments in which *C. albicans* was the challenge organism are seen in Table 2. The methodology is the same as that used in the experiments with *S. aureus*. Both BCG and *C. parvum* provided significant prolongation of the mean day of death, with little or no increase in ultimate survival.

## DISCUSSION

High doses of cyclophosphamide created an immunosuppressed state in experimental animals that is analogous to clinical situations in which there is a high risk of complicating infection. Despite a severe depression in the immunological capacity (21), BCG and *C. parvum* were capable of prolonging the survival from intravenous challenges of *S. aureus* and *C. albicans*. MER provided a similar protective effect against the *S. aureus*. Both the BCG and the *C. parvum* induced an early onset of protective capacity against *S. aureus*. However, there was a loss of efficacy in the *C. parvum* at the later periods. Live BCG was found to be the most effective. The elimination of this protective effect by isonicotinic acid hydrazide treatment and the decreased protection with lower doses of BCG suggest a requirement for an

adequate antigenic mass of BCG. This conceivably could be provided by the continued multiplication of the organisms or by a sufficient number of dead organisms. The multiplication of the live BCG in vivo may explain its prolonged duration of action as compared to the nonreplicating agents.

Although the exact mechanism of protection is not known, there is substantial evidence to indicate nonspecific enhancement of the reticuloendothelial system by the three immunostimulators tested. Macrophages in BCG-treated animals clear intravenously injected colloidal carbon at an accelerated rate (20), have increased intracellular killing of organisms (2), and contain increased concentrations of lysosomal enzymes (4). Peritoneal macrophages from BCG-treated guinea pigs have enhanced chemotactic ability and colloidal  $^{198}\text{Au}$  uptake (D. G. Poplack, N. A. Sher, S. D. Chaparas, et al., submitted for publication). The reticuloendothelial

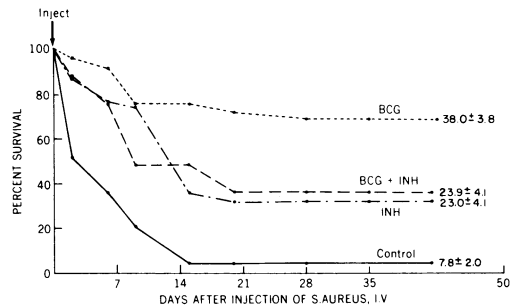


FIG. 4. Effect of isoniazid treatment on the ability of BCG to reduce mortality from *S. aureus* septicemia. Isonicotinic acid hydrazide, 0.5 mg/ml in drinking water, was administered daily starting 1 day before  $10^6$  colony-forming units of BCG. All mice received cyclophosphamide, 300 mg/kg i.p., 4 days before *S. aureus* injection on day 0. Control mice received 0.1 ml of phosphate-buffered saline. There were 25 mice in each group. The mean day of death is indicated for each group.

TABLE 1. Effect of MER pretreatment on survival from *S. aureus* septicemia<sup>a</sup>

Immunostimulant	No. of mice	Pretreatment interval (days)	% Survival by day 50	MDD ± SE (days)	P
Control <sup>b</sup>	25	14	0	3.2 ± 0.2	
MER					
0.25 mg	15	14	0	5.7 ± 0.8	<0.002
0.50 mg	15	14	20	15.3 ± 3.9	<0.002
1.0 mg	15	14	53	28.0 ± 4.8	<0.0002

<sup>a</sup> Groups of mice received various doses of MER 14 days before *S. aureus* challenge. All groups received 300 mg of cyclophosphamide per kg 4 days before the *S. aureus*. The *P* values compared the mean day of death (MDD). SE, Standard error.

<sup>b</sup> Steroid-suspending vehicle, 0.5 ml.

TABLE 2. Effect of immunostimulator pretreatment on survival from *C. albicans* septicemia

Expt	Immunostimulant	No. of mice	Pretreatment interval (days)	Dose of <i>C. albicans</i>	% Survival by day 50	MDD $\pm$ SE (days)	P
I	PBS	40	14	$2.7 \times 10^4$	0	$7.0 \pm 0$	<0.0001
	$10^6$ BCG (Brazil)	40			0	$13.4 \pm 0.3$	
II	PBS	24	21	$1.6 \times 10^3$	0	$5.4 \pm 0.5$	<0.0001
	$10^6$ BCG (Brazil)	25			0	$10.2 \pm 0.7$	
	<i>C. parvum</i> (1.0 mg)	25			24	$22.5 \pm 3.2$	

<sup>a</sup> Groups of mice received either BCG (Brazil), *C. parvum* (1.0 mg), or phosphate-buffered saline at intervals of 14 or 21 days before challenge with *C. albicans*. All groups received 300 mg of cyclophosphamide per kg 4 days before the bacterial challenge. The *P* values compared the mean day of death (MDD). SE, Standard error.

system-enhancing abilities of *C. parvum* have been well documented. One study (20) showed that *C. parvum* is comparable to BCG in non-specific stimulation of the reticuloendothelial system. Weiss et al. has shown that MER can enhance carbon clearance, increase elimination of antigen and increase levels of macrophage lysosomal enzymes (24).

The role of humoral antibodies in our model was not investigated, but Collins and Scott recently showed that *C. parvum* gave considerable protection in normal mice given lethal doses of *Salmonella enteritidis*. They found no evidence for any increases in antibody titers to the *Salmonella* (3). In a similar study with *Salmonella typhimurium*, BCG enhanced protection from infection with no effect on bactericidal antibodies (17).

Infectious complications comprise the leading cause of morbidity and mortality in patients with acute leukemia (11, 12). Postmortem examination of patients with hematologic malignancy showed that 79% had documented infections prior to death (11). Gram-negative bacteria, especially *Pseudomonas* species, as well as *C. albicans* have been increasingly the cause of septicemia and death over the last decade (9). Studies on leukemic animals, germfree from birth, show that these rodents tolerate greater dosages of radiation and chemotherapy than conventional animals since they are more resistant to infection (14).

Nonspecific immunotherapy with these preparations may reduce infections in immunodeficient patients, which may then enable these patients to undergo more intensive chemotherapy and radiation therapy. This prolonged survival of leukemic patients receiving BCG and chemotherapy may be due to such nonspecific immunostimulation (15, 22). A detailed study of the incidence of infection and the total amount

of chemotherapy received in these patients might provide further information.

The present experiments demonstrate that, under certain conditions, BCG, MER, and *C. parvum* can reduce mortality from infection in immunosuppressed animals. The mechanism of action has not been clearly defined. Whether there is a faster return to immunocompetence as a result of stimulation of cellular components of the reticuloendothelial system and circulating granulocytes remains to be determined.

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