Effect of Dose and Route of **Bacillus Calmette-Guérin** in Chemoimmunostimulation Therapy of a Murine Leukemia

J. W. Pearson, S. D. Chaparas, and M. A. Chirigos

**Introduction**

Combination and sequential drug therapy have been successful in achieving prolonged periods of remission in the treatment of neoplastic diseases. However, although the amount of cancer cell kill may be great following drug therapy, complete cures are uncommon and difficult to achieve because of the failure to eliminate totally a few residual viable tumor cells. In order to prolong and hopefully obtain permanent remission, it may be necessary to eliminate completely such neoplastic cells to prevent reinduction of the disease.

In recent years nonspecific immune stimulators have been shown alone to stimulate host factors, thus causing a reduction of tumor mass. **Corynebacterium parvum**, a highly active nonspecific stimulator of both cellular and humoral immunity, has been reported to be active against several experimental tumor systems (3, 11, 17, 18). Similarly, **BCG** has been shown alone to stimulate host factors, thus causing a reduction of tumor mass. **Corynebacterium parvum** when used in combination with Cytoxan (2). More recently, Pearson et al. (13) utilizing a murine lymphoid leukemia demonstrated a significant number of long-term survivors when **BCG** and **Corynebacterium granulosum** were used in combination with drug therapy. The present study discusses the temporal effect, dose, and route of administration of **BCG** as an adjunct to effective drug therapy.

**Materials and Methods**

**Tumor.** A Moloney lymphoid leukemia line (LSTRA) originally induced in BALB/c mice by the murine leukemia virus (Moloney), has been maintained and passaged routinely in our laboratory as a transplantable tumor line for over 300 generations in BALB/c x DBA/2 F1 (hereafter called CD2F1) mice. The ascites tumor has been serially transplanted i.p. at weekly intervals.

**Mice.** Adult CD2F1 male mice 6 to 8 weeks old were obtained from Charles River Breeding Laboratories, Wilmington, Mass. The animals were housed in plastic cages and fed Purina laboratory chow with water *ad libitum*. All animals weighed at least 23 g before they were used for experimentation.

**Drug.** BCNU was kindly supplied by the Drug Development Branch, Division of Cancer Control, National Cancer Institute, NIH, Bethesda, Md. The alkylating agent was dissolved in a steroid-suspending vehicle and administered s.c. in a constant volume of 0.01 ml/g of body weight.

**Preparation of BCG Vaccine (Mycobacterium bovis).** The Phipps strain of BCG was obtained from the Trudeau Institute Mycobacterial Culture Collection, Saranac Lake, N. Y. The organisms were grown as pellets in Long’s synthetic medium and harvested after 3 weeks of growth. Single-cell suspensions were made by suspending the pellets in Youman’s medium supplemented with 0.5% albumin and 0.05% Tween 80 and grinding gently with a Teflon grinder. The organisms were dispensed into vials, slowly

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1. The abbreviations used are: **BCG**, **Bacillus Calmette-Guérin**; **BCNU**, 1,3-bis(2-chloroethyl)-1-nitrosourea; i.d., intradermal; i.a., inguinal-axillary; f.p., footpad route.

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frozen, and stored at −70°. Colony-forming units were determined to be $2 \times 10^9$/ml in Youman's semisolid medium by the method of Rosenthal et al. (14). Total counts were determined in a Petroff-Hauser bacterial cell counter to be $8.3 \times 10^7$ organisms/ml. For use in experiments, frozen vials of BCG were thawed rapidly in a 37° water bath and diluted in phosphate-buffered saline (pH 7.0) to desired concentrations.

**Route and Site of BCG Injection.** BCG was inoculated (a) i.d. contralateral to the primary site of tumor inoculation or i.d. near the lymph nodes at the i.a. sites; (b) i.p. abdominally; (c) s.c. into each of the 4 f.p.

**Collection and Preparation of Materials for Bioassay.** Donor tissue bioassays were performed at indicated time intervals on drug-treated animals. Undiluted whole blood was obtained from donor mice and pooled; 0.2 ml was inoculated i.p. into normal recipient mice. Similarly, 0.2 ml of a 1:1 dilution of pooled spleens prepared in 0.85% phosphate-buffered saline was injected i.p. into recipient mice. All recipient mice were held for 60 days of observation before sacrifice.

**RESULTS**

We have, in previous studies, established that adult CD2F1 mice inoculated with $1 \times 10^4$ LSTRA cells die with disseminated disease within 12 to 18 days. Treatment with BCNU (30 mg/kg), at a time that the disease is systemic, results in a 10- to 12-day remission period followed by relapse and eventual death of 70 to 80% of the treated animals. The remission period was shown to extend up to the 19th day after tumor inoculation (11th day after drug treatment). We have, in previous studies, established that adult CD2F1 mice inoculated with $1 \times 10^4$ LSTRA cells die with disseminated disease within 12 to 18 days. Treatment with BCNU (30 mg/kg), at a time that the disease is systemic, results in a 10- to 12-day remission period followed by relapse and eventual death of 70 to 80% of the treated animals. The remission period was shown to extend up to the 19th day after tumor inoculation (11th day after drug treatment).

**Effect of Dose of BCG and Time of Administration.** An earlier study (13) had shown that combining BCG injection with BCNU treatment resulted in a significantly higher number of long-term survivors free of leukemia when compared to animals that received either BCG or drug alone. One variable observed in the reported study was that the effectiveness of BCG stimulation appeared to be dependent upon the time when it was administered following drug treatment. The purpose of the present study was to confirm whether a temporal effect does indeed exist and in addition whether the protective effect afforded by BCG inoculation was dependent upon the number of organisms in the inoculum. Mice were inoculated s.c. with $1 \times 10^4$ LSTRA cells on Day 0 and subsequently treated with BCNU on Day 7 for the induction of remission. Specific groups were then inoculated with a dose of viable BCG ranging from $8 \times 10^8$ to $8 \times 10^9$ on Day 10, 13, or 16 in a shaved area as described under "Materials and Methods." All animals were monitored twice weekly for tumor as well as for splenomegaly and lymphadenopathy. The results of this experiment are shown in Chart 1. There were no survivors in the untreated control group (Group 1). Approximately 20% of the animals survived with drug treatment alone when the study was terminated at 90 days (Group 2). In contrast, a substantial number of long-term survivors (50 to 90%) were obtained in all groups that received BCNU and BCG on Days 10 and 13. On the other hand, treatment with BCG on Day 16 did not produce significantly more survivors, except at the highest dose tested, than the drug-treated group alone ($p < 0.11$) (1). Survival of animals given BCG on Days 10 and 13 were independent of dose of BCG organisms over a range of 4 logs (Groups 3 to 5 and 6 to 8). For example, the inoculation of only 800 viable BCG organisms (Group 3) on Day 10 yielded 75% long-term survivors and was as effective as the group given $8 \times 10^8$ organisms (Group 5).

**Effect of Route of BCG Administration.** Since a large number of long-term survivors were obtained on Days 10 and 13 (Chart 1) following i.d. inoculation of variable doses of BCG, it was of interest to determine the effects of different doses of BCG when administered by different routes. A large group of animals was inoculated on Day 0 with $1 \times 10^4$ LSTRA cells as previously described. On Day 7, the leukemic mice were treated with BCNU, 30 mg/kg. On Days 10 and 13, specific groups of mice were given injections of different doses of BCG either by the i.p., i.a. or f.p. routes. The results of this experiment are shown in Chart 2. There were no survivors in the control group (Group 1) and approximately 25% of the animals survived with drug treatment alone (Group 2). Again, a high number of long-term survivors were obtained in all groups that received chemoimmunostimulation therapy on Day 10 or 13 when compared to the drug-treated group alone. The response appeared to be independent of dose of organisms used as well as the route of administration. Of greater significance was the observation that as few as 80 or 800 viable BCG organisms yielded a high number of long-term survivors regardless of the route or time of BCG administration (Groups 3, 4, 8, 10, 12, 13, 17, and 19). Similar results were obtained with 800 organisms administered i.d. (Chart 1, Groups 3 and 6).

**LSTRA Challenge of Long-Term Survivors.** It was important to determine whether the long-term survivors obtained through drug therapy alone or combined chemoimmunostimulation therapy would withstand a challenge of homologous LSTRA tumor cells. We considered this important for 2 reasons: (a) several "cured" animals were sacrificed for complete histological examination of all tissues and were found to be free of any leukemic cells; tissues examined were blood, bone marrow, spleen-pancreas, thymus, nodes (cervical-salivary, mesenteric, i.a.), liver, kidney-adrenal, brain, small intestine, heart-lungs and sternum; and (b) to determine whether animals that had received drug or drug plus BCG treatment had developed a specific long-lasting immunity against LSTRA. The surviving animals were maintained in individual groups according to dose of BCG and route of administration and were challenged s.c. with 2500 LSTRA cell. Normal animals of the same age groups were similarly challenged and served as controls. Since the analysis of the response of each group to the challenge was similar, regardless of BCG dose or route used, the results were pooled and are presented in Table 1. As shown in Table 1, a high percentage of animals that had received drug or drug plus BCG (Groups 1 and 2) survived or were refractory to the LSTRA challenge when compared to the control group (Group 3). Although the number of animals in Group 1 was small compared to...
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Chart 1. Combined chemoimmunostimulation therapy of LSTRA. Percentage of animals that survived for 90 days, the time at which the experiment was terminated. All animals inoculated s.c. with $1 \times 10^4$ LSTRA cells on Day ($D$) 0. Specific groups of animals treated on Day 7 with drug alone or drug plus varying doses of viable BCG given i.d. at different time intervals during the course of the disease. Each group represents 20 to 24 animals.

Chart 2. Combined chemoimmunostimulation therapy of LSTRA. Percentage of animals that survived for 90 days, the time at which the experiment was terminated. All animals inoculated s.c. with $1 \times 10^4$ LSTRA cells on Day ($D$) 0. Specific groups of animals treated on Day 7 with BCNU alone or BCNU plus varying doses of viable BCG when given on either Day 10 or 13 via 3 different routes during the course of the disease. Each group represents 12 animals.

those challenged in Group 2, there was no apparent difference between these groups in the percentage of survivors following a challenge with 2500 LSTRA cells.

DISCUSSION

The results reported here confirm and extend the findings (13) that immunostimulation therapy when used as an adjunct to effective chemotherapy under the most favorable conditions leads to a substantial number of long-term survivors free of leukemia. The most important variable in the previous studies was the time in which the nonspecific immunoenhancers were administered in relationship to drug therapy. Currie and Bagshawe (2) reported similar results against a murine fibrosarcoma in which the critical factor in their chemoimmunotherapy regimen was the time lapse between chemotherapy and the subsequent immunotherapy. The present findings not only substantiate the temporal effect exhibited by BCG but in addition show that the protection afforded by BCG inoculation was effective over a wide range of doses.

It is apparent that before successful immunotherapy may be applied the initial tumor load must be reduced. An earlier study had shown that BCG alone was ineffective against the systemic LSTRA leukemia (13). The results reported here show that the leukemia was successfully suppressed for a period of approximately 11 days with single-dose chemotherapy before relapse was detectable. However, only 20 to 25% of the animals survived with drug treatment alone. In contrast, from 50 to 100% long-term survivors were obtained in groups of animals that
received both drug and BCG. The importance of the temporal effect of BCG was demonstrated by the number of survivors obtained when the immune stimulator was administered on Days 10 and 13 (Chart 2). Approximately 50 to 90% of the animals receiving the chemoimmunostimulator on Days 10 and 13 obtained long-term survivors when the immune stimulator was administered on Day 16 did not significantly increase the percentage of survivors when compared to the group receiving BCNU treatment alone. These results further substantiate the importance of the reduced tumor load since BCNU-treated animals that received BCG on Day 16 were beginning their relapse. The effectiveness of the chemoimmunostimulation therapy was further supported by the histological observations indicating the lack of demonstrable leukemic cells in all organs examined.

The results obtained with varying doses of BCG when administered via different routes (Charts 1 and 2) were of particular significance. As few as 80 or 800 viable BCG organisms were just as effective as 8 x 10⁴ and 8 x 10⁵ organisms in producing a significant number of long-term survivors regardless of the route or time of BCG administration. It is not yet clear why such a dramatic response was obtained with such low doses of BCG following drug therapy. It is clear that the tumor mass was at low and undetectable levels when BCG was administered 3 to 6 days after BCNU treatment. It has also been suggested that BCG induces histiocytic activation (4) as well as macrophage activation (7) which may be involved as an antitumor defense mechanism. With a very low tumor load presumably only a modest level of cellular immune activation may be required for elimination of residual tumor cells to prevent relapse. This is supported by the observation in Chart 1 that show decreased protection by BCG when it is administrated 9 days past BCNU treatment, a time when the animals are entering their relapse phase.

Studies are currently underway to assess whether the use of less than 80 viable BCG organisms will also protect. Since for every viable BCG organism there are about 40 dead ones it is also important to determine what role these dead organisms play in protection against tumor.

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