Combined Chemoimmunostimulation Therapy against Murine Leukemia


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SUMMARY

The effect of combining chemotherapy and immunostimulation against a murine lymphoid leukemic line designated as LSTRA was investigated. Inoculation of $1 \times 10^6$ ascites cells s.c. resulted in the death of all animals within 12 to 15 days. Treatment of infected mice with 1,3-bis(2-chloroethyl)-1-nitrosourea, 20 mg/kg, increased the range of death to 20 to 28 days with a significant number of long-term survivors (12%). The parameters utilized to determine the appearance of disease, induction of remission, and relapse following drug therapy were based on the bioassay of donor tissue at specified time intervals. When Bacillus Calmette-Guérin and Corynebacterium granulosum were used following drug administration of 1,3-bis(2-chloroethyl)-1-nitrosourea, 20 mg/kg, a high percentage of long-term survivors occurred in contrast to the groups of animals treated with immunostimulators or drug alone.

The most important variable in these studies was the time in which immunostimulants were given following drug therapy. C. granulosum, when given 3 days after 1,3-bis(2-chloroethyl)-1-nitrosourea therapy, resulted in 76% complete remissions, while B. Calmette-Guérin was most effective 12 days after drug treatment, yielding approximately 68% long-term survivors. The implications of these results to cancer therapy are discussed.

INTRODUCTION

The control of cancers through the application of drugs, surgery, or radiation, used alone or in combination, generally has not been effective in producing a high percentage of long-term survivors. The demonstration of tumor-specific antigens on cells derived from human and animal cancers (4, 5, 11), however, suggests that immunological control measures might be valuable adjuncts to these forms of therapy. One possible approach to this problem is the use of nonspecific immunoenhancers.

BCG\(^1\) has previously been reported to protect against the induction of tumors induced with chemical and viral-induced transplantable cells (10) and to inhibit viral oncornogenesis (6, 12, 13). Recently, Zbar et al. (16) reported complete tumor inhibition in guinea pigs when infection with living BCG occurred at the site of tumor inoculation. In addition, these animals resisted a subsequent challenge of viable tumor cells. Mathé et al. (7) and Morton et al. (8) have also reported the effectiveness of BCG in the treatment of human cancers.

Similar findings by use of certain species of Corynebacterium as adjuvants have been reported in the treatment of experimental tumors (3, 15). The present study was designed to evaluate the effects of BCG and C. granulosum when they are used in combination with drug therapy. The results suggest that these immunostimulators may prove to be valuable adjuncts to chemotherapy in the control of cancers.

MATERIALS AND METHODS

Tumor. A Moloney lymphoid leukemic 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 CDF\(_1\) mice. The ascitic tumor is serially transplanted i.p. at weekly intervals.

Mice. Adult CDF\(_1\) males, 8 to 12 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.

Drug. BCNU was kindly supplied by the Cancer Chemotherapy National Service Center, 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.

Nonspecific Immunostimulators. The Glaxo freeze-dried BCG, a nonviral form of the tubercle bacillus, was obtained from Eli Lilly and Company, Indianapolis, Ind. The BCG was prepared by resuspension of the lyophilized material in each vial to final volume of 0.4 ml by use of 0.85% phosphate-buffered saline. Mice were inoculated intradermally with approximately 2 to $4 \times 10^4$ organisms/0.2 ml.

C. granulosum was prepared by heating the bacteria at 60\(^0\) for 1 hr in the presence of 2% formalin. Animals received 100 $\mu$g/0.2 ml intradermally.

Collection and Preparation of Materials for Bioassay. Donor tissue bioassays were performed at indicated time intervals on both infected and drug-treated animals. Undiluted whole blood was obtained from mice, pooled, and inoculated i.p. into normal recipient mice, 0.2 ml/mouse. Similarly, 0.2 ml of a 50% concentration of pooled spleens prepared in 0.85% phosphate-buffered saline was injected s.c. in the right inguinal region of the recipient mice. All recipient mice were held for 60 days before sacrifice.
RESULTS

Onset of Disease. The objective of this study was to determine the time after LSTRA inoculation when the leukemia could be first detected systemically. Twenty-five donor mice were given s.c. injections of $1 \times 10^4$ LSTRA cells, a cell concentration that induces a rapidly growing tumor at the site of inoculation and subsequent development of splenomegaly and lymphadenopathy. All animals succumb to the leukemia within 12 to 15 days. At 3, 5, 7, 9, and 11 days after inoculation, 5 mice were sacrificed and their blood and spleens were collected. Bioassays of the whole blood and spleens were carried out as described under "Materials and Methods." Results in Chart 1 show that the disease was transmitted to all recipient mice inoculated with blood or spleens from donor mice sacrificed 7 days after tumor inoculation. Tumors (5 to 7 mm) also were detectable at the site of inoculation by the 7th day. The earlier occurrence of death of recipient mice inoculated with equivalent preparations of blood and spleens from donor mice sacrificed on the 9th and 11th day indicates the intensity of the disease prior to death.

Drug Therapy and Induction of Remission. The purpose of this study was 2-fold: (a) to find a drug dose active against LSTRA and (b) to determine the period of remission following drug therapy. Test groups containing 15 mice each were inoculated s.c. with $1 \times 10^4$ LSTRA cells. On Day 7, when the disease was systemic (see Chart 1), individual groups of mice were treated with varying concentrations of BCNU and followed for survival. These results are presented in Table 1.

BCNU was very effective against LSTRA, regardless of the dose of drug administered, when compared to the untreated control group. The range of death in the treated groups was protracted over a longer period of time (20 to 35 days as compared to 12 to 15 days in the control group) and the first deaths did not occur until all the control mice were dead. A single treatment with 30 mg/kg of BCNU on Day 7 resulted in 40 to 50% survivors when the experiment was terminated at 60 days. However, at this dose, detectability of disease was noted on the basis of body weight loss as well as occasional nontumor death. In contrast, groups of mice receiving a single injection of BCNU, 25 or 20 mg/kg, (Groups 3 and 4) exhibited no apparent signs of toxicity. Long-term survivors were also obtained in these groups.

A 2nd observation shown graphically in Chart 2 is the time of relapse following drug therapy. Three days after treatment with BCNU, 20 mg/kg, (Day 10 posttumor inoculation), and every other day thereafter up to 22 days postinoculation, mice treated with BCNU were sacrificed and whole blood and spleen were bioassayed into normal recipient mice. Systemic disease was not again detected until 16 to 18 days following LSTRA inoculation. Thus, a remission period was attained in which no disease was

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Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>LSTRA at Day 0 (x 10^4)</th>
<th>BCNU at Day 7 (mg/kg)</th>
<th>Range of death (days)</th>
<th>Survival at 60 days (%)</th>
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<td>30</td>
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<td>1</td>
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<td>1</td>
<td>20</td>
<td>22-31</td>
<td>30-40</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>1</td>
<td>20</td>
<td>20-28</td>
<td>10-20</td>
</tr>
</tbody>
</table>

*a Mice inoculated with $1 \times 10^4$ LSTRA cells on Day 0.

*b One treatment of BCNU given on Day 7.

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Chart 1. Transmission of LSTRA leukemia. Donor mice inoculated with $1 \times 10^4$ LSTRA cells on Day 0. Donor mice sacrificed on indicated days, and blood and spleen bioassayed in recipient mice (6 mice/group). Number above each column, % of survivors at 60 days when experiment was terminated.

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Chart 2. Transmission of LSTRA leukemia after drug treatment. Donor mice inoculated with $1 \times 10^4$ LSTRA cells on Day 0. All mice received 1 injection of BCNU on Day 7, when disease was systemic. Donor mice sacrificed on indicated days, and blood and spleen bioassayed in recipient mice (6 mice/group).
detectable by bioassay criteria. This was followed by a partial and then full relapse on the 9th to 13th day following drug therapy.

Combined Chemoinmunostimulation Therapy. These studies were designed to test the possibility that the use of nonspecific immunological stimulators such as BCG or *Corynebacterium granulosum* might prolong the period of remission and/or increase the number of survivors when applied following therapy with BCNU, 20 mg/kg.

In this study, a large number of mice were given s.c. injections of $1 \times 10^4$ tumor cells on Day 0. Seven days later, when the disease was systemic, specific groups of mice were treated with drug or stimulators used alone or in combination. When combinations were given, BCG and *Corynebacterium granulosum* were administered intradermally on Days 10, 13, 16, or 19 following BCNU therapy on Day 7. The adjuvants were given in a shaved area on the opposite side from that in which the tumor cells were initially injected. All animals were monitored twice weekly for tumor as well as for splenomegaly and lymphadenopathy. The results of this experiment are shown in Chart 3. The range of individual deaths in the untreated controls was 11 to 16 days, with a median survival time of 12.0 days. Treatment with BCNU, 20 mg/kg, on Day 7 resulted in a 100% increase in median survival time (24 days); and the range of death was protracted over a longer period of time, with 1 animal still alive when the experiment was terminated on Day 85. The median survival time of mice treated alone with BCG or *Corynebacterium granulosum* on Day 7 did not differ markedly from that of the untreated control group (16.0 and 14.0 days, respectively). Substantial numbers of long-term survivors were noted in groups that received combined chemoinmunostimulant therapy. This effect was most pronounced with BCG in drug-treated mice when administered on Day 19, at which time the animals were in a state of remission (see Chart 2). When the experiment was terminated on Day 85, 60% of the mice were alive in this group. In addition, at autopsy 85 days after tumor inoculation, all animals appeared to be free of disease. BCG treatment on Day 13 or Day 16, when the drug-treated animals were in partial remission, resulted in a protracted life-span.

![Chart 3. Combined chemoimmunostimulant therapy of LSTRA. BCNU and stimulator given alone or in combination at specified time intervals following s.c. inoculation of $1 \times 10^4$ LSTRA cells on Day (D) 0. Each point represents time of death of individual animal (15 mice/group). Columns, median survival time for each group. Coryn. Gran., Corynebacterium granulosum.](image)

*C. granulosum* was also effective following BCNU treatment. In contrast to BCG, however, *C. granulosum* was most effective 3 days after BCNU therapy (Chart 3), at which time the animals were in a state of remission. Ten out of 15 animals (66%) were still surviving when the experiment was terminated on Day 85. When *C. granulosum* was given at later time intervals (Days 13, 16, or 19), a higher number of long-term survivors was observed in these groups when compared to mice treated with drug alone.

![Chart 4. Combined chemostimulant therapy of LSTRA. A composite of 3 experiments demonstrating total number of survivors, 85 days after tumor inoculation, in groups treated with drug or adjuvant alone or in combination at specified times following s.c. inoculation of $1 \times 10^4$ LSTRA cells on Day (D) 0. Coryn. Gran., Corynebacterium granulosum.](image)

Chart 4 summarizes a composite of 3 experiments that demonstrate the total number of survivors in groups treated with drug or adjuvant alone or in combination. There were no survivors in the untreated control group or in groups treated with BCNU or *Corynebacterium granulosum* alone. Approximately 12% of the animals survived with drug treatment alone. Administration of BCNU 12 days (Day 19) after drug therapy resulted in approximately 68% survivors, thus substantiating the importance of the time interval between BCG treatment and drug therapy. Earlier treatment with BCG also resulted in a slightly larger number of survivors when compared with the drug-treated group, but the effect was not as pronounced as when BCG was administered late.

The effect of early treatment with *C. granulosum* following BCNU therapy is also apparent in Chart 4. Following combination therapy, 76% of the animals survived when *C. granulosum* was administered 3 days (Day 10) after drug therapy. Treatment with this adjuvant at later time intervals also resulted in a slightly increased number of long-term survivors when compared to the drug-treated controls.

DISCUSSION

Recent reports demonstrate that nonspecific immune stimulation with *Corynebacterium parvum* significantly inhibited mouse mammary carcinoma (14) and a transplanted methylcholanthrene-induced fibrosarcoma (3). Protection against a guinea pig hepatoma with BCG has also been reported (16). BCG has been shown to afford excellent
protection against tumor induction by the murine sarcoma virus (Moloney) (12).

In all of these reports, control of tumor was based on the stimulation of host immunity. The exciting results reported by Mathé et al. (7) in the treatment of acute lymphoblastic leukemia in children suggest that such nonspecific stimulators may be usefully combined with conventional chemotherapy. Combined chemoimmunotherapy is indeed proving to be a more active means of controlling experimental tumors than either treatment alone. When given to mice bearing established tumors, C. parvum, a highly active nonspecific stimulator of both cellular and humoral immunity, stimulated host factors, thus causing reduction or disappearance of tumor mass (3, 9, 15). Currie and Bagshawe (2) reported that the antitumor effect achieved with Cyttoxan against established murine fibrosarcoma grown in syngeneic mice was markedly improved by active immunotherapy with C. parvum. The most critical factor in the chemoimmunotherapy regimen reported was the time lapse between the chemotherapy and the subsequent immunotherapy. A similar response of active immunotherapy preceded by cytoreductive chemotherapy was reported by Amiel and Berardet (1).

The results presented in this report confirm and extend the finding that combining treatment with an effective antitumor drug with a nonspecific immune stimulator is more effective than either treatment alone. Suppression of LSTRA leukemia, after single-dose chemotherapy, was obtained for a period of 8 to 10 days but produced relatively few long-term survivors. Stimulation with BCG or C. granulosum alone was ineffective. In no case was either of these agents significantly curative, but when they were combined under the most favorable conditions a substantial number of complete and lasting remissions was obtained. The most crucial variable in the use of this combination appears to be the interval between the single-dose chemotherapy and the subsequent nonspecific immune stimulation. It is not yet clear why BCG was most active when given at a later interval than C. granulosum. Repeated injections of BCG or C. granulosum did not improve the response (unpublished observations), and this finding is in agreement with results reported by Currie and Bagshawe (2). The effectiveness of BCG at the time of relapse, a time when increasing amounts of antigen are present, suggests that BCG is dramatically enhancing a tumor-specific response as well as activating nonspecific elements of the reticuloendothelial system. In contrast, the results suggest that C. granulosum may not be as effective in enhancing the tumor-specific response but may be acting primarily on nonspecific elements of the reticuloendothelial system. C. granulosum was reported to stimulate specifically the phagocytic activity of the reticuloendothelial system (14). BCNU, like many alkylating drugs, is an immunosuppressive agent (J. P. Glynn, personal communication); however, the suppressive activity, if present, did not alter the activity of immune stimulation by C. granulosum when given on the 3rd day after drug treatment. Thus, drug immunosuppression is an unlikely explanation for the difference in time interval of effectiveness for BCG or C. granulosum.

The effectiveness of the chemoimmunotherapy approach may depend on many factors, such as antigenicity of the tumor; rate of tumor cell replication before and after drug therapy; the immune status of the host at entry into this therapy regimen, but particularly after drug therapy; selection of the most effective stimulant; and the type of chemotherapy necessary to retard the tumor. Despite the many variables involved, the successes achieved are encouraging. A further assessment of the potential value of active immunotherapy as an adjunct to the more conventional forms of cancer treatment must be pursued, particularly in other viral and/or spontaneous tumor systems. The oncornavirus-induced and spontaneously occurring feline leukemia and sarcomas and the spontaneous canine tumors afford excellent model systems for further assessment and hopefully eventual application to man.

REFERENCES

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