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Prolongation of Chemotherapeutically Induced Remission of a Syngeneic Murine Leukemia by L-2,3,5,6-Tetrahydro-6-phenylimidazo[2,1-*b*]thiazole Hydrochloride

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SUMMARY

L-2,3,5,6-Tetrahydro-6-phenylimidazo[2,1-*b*]thiazole hydrochloride (LMS), when used in concert with 1,3-bis(2-chloroethyl)-1-nitrosourea, resulted in a significantly higher percentage of long-term leukemic-free survivors. The additive effect provided by LMS treatment was evident during the immunosuppressed period induced by 1,3-bis(2-chloroethyl)-1-nitrosourea treatment and when tumor load was minimal. Treatment with LMS alone did not appear to possess any significant antitumor effect.

The beneficial effect of LMS treatment may be attributable to the immunostimulatory activity reported for this drug. LMS appears to possess characteristics that make it an excellent candidate for use as an immunostimulant in cancer combined modality treatment.

INTRODUCTION

In recent years, the application of nonspecific immune stimulators, when used alone or combined with other forms of therapy to augment host immunity, suggests that immunological control measures may prove valuable adjuncts to the control of neoplasia.

Bacillus Calmette-Guérin affords protection against tumors induced with chemicals (28) or viral-transplantable cells (13, 15) and inhibits viral oncogenesis (7, 8, 22, 23). *Bacillus Calmette-Guérin* has also been reported to be effective in controlling human leukemias (11, 12) and malignant melanomas (10).

The use of chemicals known to stimulate cellular or humoral immune responses (1, 6, 14, 25) has not been investigated to a great extent to determine the effect of the chemicals when used in concert with chemotherapy in the treatment of leukemia. LMS[1] reportedly has a stimulatory effect on antibacterial immunization (19), on antibody-forming cells (21), and in enhancing the graft-*versus*-host reaction (20). Of particular significance are the reports of LMS acting as an immunostimulant in man (2, 5, 24, 26). We recently reported the beneficial effect of LMS when used in concert with an effective remission-inducing drug (4). The results of this study showed that, with the murine LSTRA-transplantable lymphoid leukemia, when LMS was used following 1 administration of BCNU, a significantly higher percentage of long-term survivors was attained.

In the present study, we used a different syngeneic tumor and determined the effect of LMS when used in combination with BCNU. The objectives of the experiment were to determine whether a similar beneficial effect could be achieved with a different tumor system and to assess what effect the immunosuppressive effect of BCNU treatment had on the cellular immune response.

MATERIALS AND METHODS

Animals. Adult BALB/c × DBA/2 (hereafter called $CD2F_1$) male mice, 6 to 8 weeks old, were supplied by the Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md. 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.

Tumor. A Moloney lymphoid leukemia line (MCAS-10), originally induced in $CD2F_1$ mice by inoculation with the Moloney murine leukemia virus, has been maintained in $CD2F_1$ mice and passaged routinely in our laboratory in the ascites form for >600 generations. The ascites tumor is passed i.p. at weekly intervals.

The MBL-2 line, a Moloney lymphoid leukemia line originally induced in C57BL/6 mice by inoculation with the Moloney murine leukemia virus, was kindly supplied by Dr. D. Houchens, Drug Evaluation Branch, Division of Cancer Treatment, National Cancer Institute and passaged routinely in our laboratory in the ascites form for >86 passages. The ascites tumor is passed i.p. at weekly intervals.

Drug. BCNU was kindly supplied by the Drug Development Branch, Division of Cancer Control, National Cancer Institute, NIH. The alkylating agent was dissolved in hydroxypropyl cellulose (0.3% solution) and administered in the scapulary area in a constant volume of 0.01 ml/g of body weight.

LMS was kindly supplied by Dr. P. Janssen, Janssen

[1] The abbreviations used are: LMS, L-2,3,5,6,-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole hydrochloride; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MST, median survival time.

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Pharmaceutica, Beerse, Belgium. The chemical was dissolved in sterile 0.85% NaCl solution and administered i.p. in a constant volume of 0.01 ml/g of body weight.

Homograft Response. MBL-2 ascites cells (1×10^6 cells) were inoculated s.c. in the right inguinal region of $CD2F_1$ mice, and tumor palpations, were conducted every 3rd day until regression occurred. In studies to determine abrogation of the homograft response, mice were treated with BCNU, at 30 mg/kg, s.c. in the scapular area 1, 2, 4, 6, 8, or 10 days before MBL-2 tumor inoculation.

Collection and Preparation of Materials for Bioassay. The bioassay procedure for determining onset of disease, remission, and relapse following drug therapy has been reported previously (16). All recipient mice were observed for 60 days for signs of the disease.

RESULTS

Onset of Disease, Induction of Remission, and Relapse. The study was designed to determine when systemic leukemia could be first detected after MCAS-10 inoculation and how long BCNU-induced remission would last before relapse occurred. Chart 1 is a composite of 4 experiments conducted at different times: 2 experiments were conducted to establish the period when systemic leukemia is detectable by bioassay technique (*Control*) and 2 experiments demonstrated the period of remission and relapse (*Treated*).

Large numbers of donor mice were inoculated s.c. with 1×10^4 MCAS-10 tumor cells on Day 0. Grossly, the 1st manifestation of tumor growth occurred at the inoculation site by the 7th day; then splenomegaly and lymphadenopathy developed, and within 14 to 20 days all animals died with leukemia (MST, 15 days). At 9 and 12 days after tumor inoculation, 6 mice were sacrificed, and whole blood or spleen brei was bioassayed i.p. into recipient mice. As shown in Chart 1, the leukemia was readily transmissible to all recipient mice from donor blood and spleen by the 9th day, and the progressive nature of the disease is further demonstrated by the earlier death of recipient mice inoculated with blood and spleen from donors sacrificed by the 12th day.

To test the length of the remission period induced by BCNU treatment, similar experiments were conducted. Large numbers of mice were inoculated on Day 0 with 1×10^4 MCAS-10 tumor cells. On the 9th day, when the disease was systemic, animals were treated with BCNU, 30 mg/kg, and at different time intervals treated mice were killed and whole blood and spleen were bioassayed in recipient mice (Chart 1, *Treated*). Remission induction had begun by the 12th day (3 days after treatment), as evidenced by the longer survival time of the recipients inoculated with blood or spleen from treated animals as compared to controls. By the 15th day remission appeared complete, disease was not transmissible to recipients, and treated mice remained in remission until the 22nd day (13 days after drug treatment). Relapse was evident by the 26th day, with all recipient mice showing signs of the transmitted disease. The induction, remission, and relapse periods are also shown in Chart 3 in conjunction with the response attained in the homograft response study.

Effect of Combined BCNU and LMS Treatment. The MCAS-10 leukemia system was used (Chart 2) to confirm and extend the observation that LMS augments BCNU therapy (4).

BCNU therapy was withheld until systemic leukemia was established. All tumor control mice died within 11 to 20 days (MST 15 days). BCNU treatment significantly extended survival time (MST, 52 days) with 33% of the animals alive and tumor free at 160 days when the experiments were terminated. The additional treatment with LMS during early remission (Day 12, Groups 3 and 4) and remission period (Day 15, Groups 5 and 6) resulted in a more extended survival period and a larger number of tumor-free survivors. Treatment with LMS, 5 mg/kg, appeared to be as effective as with the 20 mg/kg dose at these 2 time periods (Day 12 or 15). However, treatment

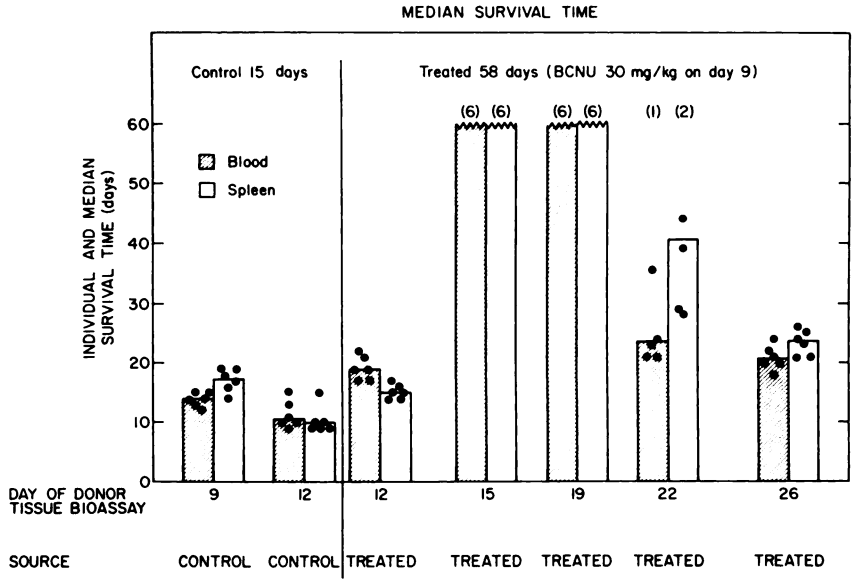

Chart 1. Transmission of MCAS-10 leukemia. Tumor-inoculated control (nontreated) and treated mice were sacrificed on indicated days, and blood and spleen were bioassayed in recipient mice (6 mice/group). *Numbers in parentheses*, number of survivors at 60 days when experiment was terminated.

with LMS just prior to the relapse period (Day 19, Groups 7 and 8) was effective only at the higher dose of 20 mg/kg (Group 8). In contrast to the 33% survival attained with BCNU alone (Group 2), the lower dose of LMS (Group 7) was ineffective and resulted in only a 25% survival.

Two groups of mice were treated with LMS alone as comparative control groups to the untreated (Group 1) and BCNU-treated mice (Group 2) to assess whether LMS possessed any antitumor activity. Five daily treatments with LMS, 5 mg/kg (Group 10), did not exert demonstrable antitumor effect. All animals died within the same time period as the untreated tumor control animals (Group 1). A single treatment of 20 mg/kg on Day 9 did cause a slight but not significant increase in MST and a spread in death pattern. Sixteen % of the animals survived, free of tumor. However, we have observed this survival in only 1 of 3 experiments, although the extended death pattern has been observed consistently.

Duration of Immunosuppression Resulting from BCNU Treatment. To evaluate whether BCNU is, indeed, immunosuppressive and for what period, suppression of the homograft response was tested by pretreatment with BCNU. The test was based on the suppression of the homograft response to the MBL-2 leukemic cells in drug-pretreated $CD2F_1$ mice. Any potential antitumor effect was avoided by withholding tumor inoculation until BCNU treatment was completed. No residual antitumor effect was anticipated since it has been reported that the duration of an effective concentration of BCNU in mice is 1 hr or less (3).

Results in Table 1 and Chart 3 show that the homograft response was abrogated as a result of pretreatment with BCNU, 30 mg/kg. All nontreated control animals rejected

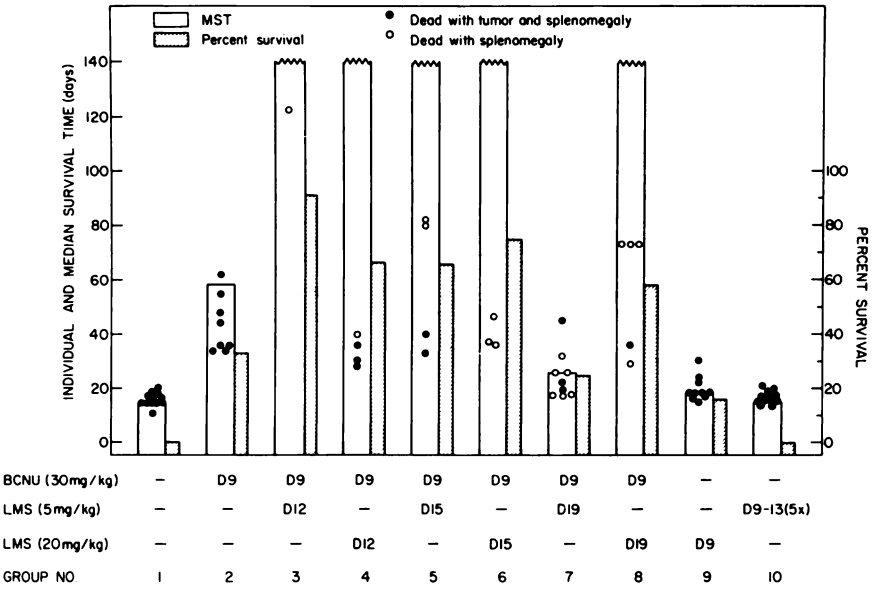

Chart 2. Combined modality therapy of MCAS-10. Single or combined drug treatment at specified time intervals following tumor inoculation on Day (*D*) 0. *Points*, time of death of individual animals (12 mice/group).

Table 1

Measure of immunosuppressive effect of BCNU treatment

All mice were inoculated with 1×10^8 MBL-2 ascites cells on Day 0. Ten mice per group.

	BCNU, 30 mg/kg																				
	None			Day −10			Day −8			Day −6			Day −4			Day −2			Day −1		
Day of observation	T/T[a] (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)	T/T (%)	ATS (mm)	R (%)
5	100	4		100	4		100	4		100	4		90	3		100	4		100	4	
8	100	6		100	6		100	6		100	5		100	6		100	6		100	6	
11	90	4	10	100	4		100	4		100	7		100	10		100	12		100	10	
13	60	3	40	70	3	30	90	4	10	100	7		100	12		100	14		100	14	
18	0		100	0		100	20	3	80	90	7	10	100	14		100	20		100	21	
21							0		100	60	9	40	100	17		100	18		100	23	
25										60	12	40	100	21		100	24		100	26	
29										50	22	50	100	24		100	25		100	29	
33										40	25	60	100	22	0	100	28	0	100	25	0
% D/T	0			0			0			40			100			100			100		

[a] T/T, mice with tumor/total number of mice; ATS, average tumor size of mice with tumor; R, regression of tumors; D/T, percentage of mice dying with tumor.

the homograft within 18 days (Table 1). In contrast, when BCNU was administered up to 4 days prior to tumor inoculation, all animals developed progressively growing tumors without any rejection occurring. Treatment 6 days prior to tumor inoculation resulted in only 40% death with tumor and 60% regression. Treatment 10 or 8 days prior to tumor inoculation resulted in only a slight delay in homograft rejection.

These results indicate that the immunosuppressive effect of BCNU was of a 6-day duration.

LMS, when used as an adjuvant with BCNU treatment (Chart 2), was most effective when administered 3 or 6 days after BCNU treatment, a time when remission induction was initiated (Chart 1, 3 days posttreatment) and complete (Chart 1, 6 days posttreatment). However, the immunological competence of the host to reject a homograft was suppressed up to 6 days, due to the immunosuppressive effect of BCNU treatment (Table 1). A composite of the results shown in Chart 1 and Table 1 is reproduced in Chart 3.

The results indicate that LMS was effective when administered at a time when (*a*) the mice were in the early remission period but immunoincompetent (Day 12); (*b*) when the mice were in remission and partially competent (Day 15); and (*c*) when the mice were in complete remission and fully competent (Day 19).

DISCUSSION

Results of this study confirm and extend the observation that LMS, a reported humoral and cellular immune stimulator, when used in conjunction with an effective chemotherapeutic agent, results in an augmented therapeutic response. The effectiveness of LMS was most pronounced when it was administered during the remission induction phase and early remission period. When LMS treatment was delayed until late in the remission period, a higher dose of LMS was required.

The majority of the animals that died after receiving the combined treatment had no evidence of a recurrence of the local s.c. tumor. This was also evident when treatment with LMS was delayed until just prior to relapse. In contrast, all animals that eventually died after receiving BCNU treatment alone expired with recurring local tumor. At present, we do not have a plausible explanation for this phenomenon.

The data reported here suggest that the homograft response to MBL-2 leukemia may be used as a model system for testing agents that may interfere with the immune response. BCNU, at the dose tested, appears to suppress the immune response to the MBL-2 homograft for a period of 6 days.

The mechanism by which LMS exerted its additive protective effect cannot be ascribed to any antitumor effect, since results from the present study and a similar study (18) indicate that LMS possesses no antitumor activity. Several reports indicate that LMS acts as an immunostimulator (2, 4, 5, 19–21, 24, 26). *In vitro* studies indicate that LMS stimulates the T-cells (9, 27). Stimulation of this type of cell would be expected to provide the host with increased numbers of potentially specifically reactive cells in early stages of chemotherapeutically induced remission, where the number of tumor cells is at a minimum. Histological examination of spleens from leukemic mice treated with BCNU and LMS shows an earlier return of lymphoid cells in the splenic lymphoid follicles and lymphoid elements in the red pulp (17).

It appears that the stimulatory effect exerted by LMS treatment acts in concert with the remission-inducing effect of BCNU. Its effectiveness is maximal at a time when remission of the disease occurs and, coincidentally, during the immunosuppressive period. These results suggest that LMS stimulation of the immune response could be primarily due to an initial nonspecific proliferation of lymphoid cells, leading to an increased number of cells capable of reacting specifically to the tumor antigen. Such a mechanism would be useful in overcoming depleted immune cell

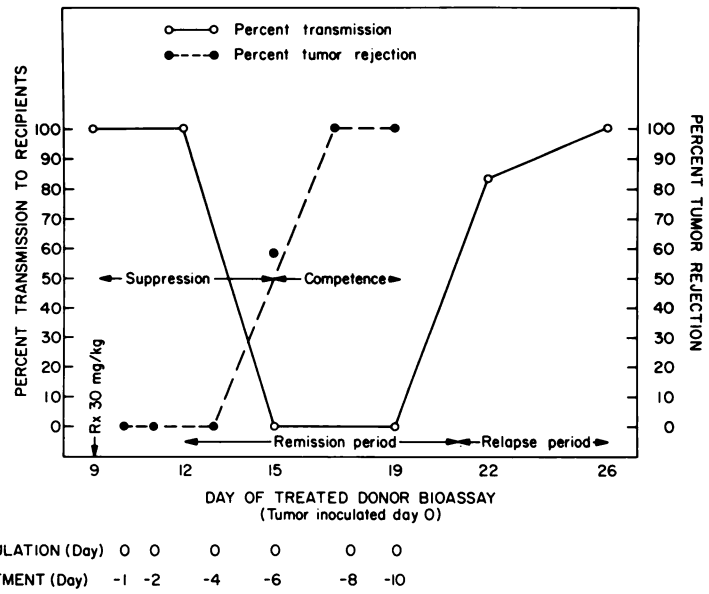

Chart 3. Composite of Chart 1 and Table 1. Superimposing remission induction attained by BCNU treatment (Chart 1) and duration of suppression of the homograft response induced by BCNU (Table 1).

compartments in immunosuppressed animals, allowing restoration of immunosurveillance in depression due to cancer. Indeed, Tripodi *et al.* (24) have demonstrated that LMS stimulates immunoresponses in anergic tumor patients. LMS appears to possess characteristics that make it an excellent candidate for use as an immunostimulant in cancer combined modality therapy.

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