Elimination of EL-4 and L1210 Murine Tumors by 1,3-bis (2-Chloroethyl)-1-Nitrosourea Requires an Intact Immune Response (43992)

TIMOTHY A. STEELE[1] AND DONALD C. COX
Department of Microbiology, Miami University, Oxford, Ohio 45056

Abstract. **The chemotherapeutic agent 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) is commonly used to treat several types of human cancers. Recent investigations have suggested that elimination of tumor by BCNU is dependent on more than the cytotoxic activity of the drug. We have extended those findings by showing that cyclosporin A (CS) can inhibit the BCNU-mediated rejection of EL-4 or L1210 tumors in mice. It was shown that mice could be cured of EL-4 or L1210 ascites tumors with a single intraperitoneal injection of BCNU. When CS, an inhibitor of the activation of T lymphocytes, was administered to mice that had received either EL-4 or L1210 tumor and were treated with BCNU, nearly all the mice died by Day 60. When CS was administered to BCNU-treated mice starting at 1, 2, or 3 weeks after tumor injection, inhibition of the BCNU therapy did not occur. Finally, the ability of animals that had been cured of tumor by the BCNU therapy to reject a lethal challenge dose of homologous tumor was shown to be CS insensitive. These results suggest that the BCNU-mediated elimination of tumor from mice requires a functional immune response in addition to the cytotoxic activity of this chemotherapeutic agent.**

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During the course of studies investigating the rejection of EL-4 tumor using the combined modalities of low-dose 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) and reovirus type 3, it was shown that tumor-bearing mice treated with 9 mg/kg BCNU resulted in 20% survival, whereas 100% of BCNU-treated tumor-bearing mice receiving daily intraperitoneal (ip) injections of cyclosporin A (CS) died (1). It is important to note that the dose of BCNU was chosen to yield only low levels of survival in the above studies. However, these results suggested that BCNU may require an intact immune response to eliminate fully host tumor. In order to determine whether an immune response was required in the BCNU-mediated rejection of tumor, we undertook the present set of investigations.

BCNU is an alkylating agent that has been shown to be effective in the chemotherapy of human tumors including Hodgkin's disease, non-Hodgkin's lymphoma, brain tumors, and multiple myeloma (2). In addition to its direct action of cytolytic tumor reduction, evidence in mice suggests that BCNU may also require a functional immune response directed to the tumor for complete tumor eradication to occur. T suppressor cell activity has been shown to be inhibited by BCNU, thus potentially allowing an effective antitumor immune response to occur (3). BCNU-treated tumor-bearing mice also had enhanced levels of lymphokine-activated killer cell activity and cytotoxic T lymphocyte activity against tumor (4). In addition, *in vitro*

[1] To whom requests for reprints should be addressed at his current address: Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, GA 31207-0001.

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treatment of tumor cells with BCNU allowed macrophages to destroy tumor cells more effectively (4).

Cyclosporin A is a potent immunosuppressive agent that is the current drug of choice in human heart, liver, and kidney transplantation (5). CS exerts a suppressive effect on T cell activation by preventing the production and release of interleukin-2 and other required lymphokines (6). The drug has been used *in vivo* to explore the role of the immune response in the T cell–mediated immunopathologic conditions induced by lymphocytic choriomeningitis virus (7) and Borna disease virus (8). The immunosuppressive qualities of CS make it a useful agent to study *in vivo* immune responses, and in the present studies CS was utilized to investigate whether a host immune response plays an obligatory role in the BCNU-mediated eradication of tumor.

Materials and Methods

Mice. Male B6D2F1 (C57BL × DBA/2) mice and female CD2F1 (BALB/c × DBA/2) mice (Harlan-Sprague Dawley, Indianapolis, IN) had unlimited access to food and were housed and cared for according to University of Wisconsin—Milwaukee Animal Care and Use Committee guidelines and *Principles of Laboratory Animal Care* (National Institutes of Health publication No. 85–23, revised 1985). The mice were 7–12 weeks of age and weighed 20–28 g each.

BCNU Therapy. BCNU (Bristol-Myers, Evansville, IN), in powdered form, was dissolved in absolute ethanol and diluted to 1.5 mg/ml in sterile distilled water and 0.85% saline. Dilutions of this stock solution were used to provide the desired dose of BCNU, which ranged from 0 to 30 mg/kg body wt. Control mice received phosphate-buffered saline solution containing 0.3% absolute ethanol.

Male B6D2F1 mice or female CD2F1 mice were injected intraperitoneally (ip) with 1×10^5 EL-4 or 1×10^5 L1210 tumor cells (American Type Culture Collection, Rockville, MD) in 0.2 ml of phosphate-buffered saline, respectively. The tumor cells grew as an ascites tumor and were syngeneic for the respective mouse strain. Four days after tumor injection, BCNU at the indicated dose was injected into the tumor site. Tumor control animals received phosphate-buffered saline in place of BCNU. Survival for each group of animals was followed for 60 days after tumor injection. Surviving animals were challenged with 1×10^5 homologous EL-4 or L1210 cells. Survival of challenged animals was monitored for 30 days. Experiments normally included 10 animals in each group.

Tumor Cells. EL-4 lymphoma and L1210 leukemia cells were serially passaged every 10–14 days ip in B6D2F1 male mice or CD2F1 female mice, respectively, as an ascites tumor. Cells were removed by peritoneal lavage and treated with 0.14 *M* Tris-buffered ammonium chloride to remove red cells. The cells were washed three times in phosphate-buffered saline and adjusted to a concentration of 5×10^5 cells/ml. Cell viability was monitored by trypan blue exclusion and was typically found to be greater than 95% viable.

Cyclosporin A. CS was a kind gift of Sandoz Research Institute (Hanover, NJ). Cyclosporin A, in the supplied injectable form, was diluted in phosphate-buffered saline solution to allow a final dosage for each mouse of 30 mg/kg administered starting at Day −1 relative to tumor administration, unless otherwise specified, and continued daily for 21 days. The concentration was adjusted to allow the ip injection of a total volume of 0.2 ml/mouse.

Results

Determination of the Dose of BCNU Required to Eradicate EL-4 Tumor. Because various tumor cell lines differ in their sensitivity to chemotherapy, it was important to determine the effect of various doses of BCNU on EL-4 tumor rejection *in vivo*. Tumor-bearing mice were injected ip with varying quantities of BCNU and monitored for survival. As shown in Figure 1, it was determined that 50% survival occurred with 20 mg/kg BCNU and that 30 mg/kg BCNU produced 90% survival. All mice that survived to 60 days post–tumor injection exhibited no apparent morbidity and were considered cured of tumor. Doses of BCNU greater than 35 mg/kg were found to be toxic to the mice, with deaths unrelated to the tumor burden occurring (data not shown). Since it provided consistently high levels of survival, 30 mg/kg BCNU was chosen for all subsequent experiments involving EL-4 tumor.

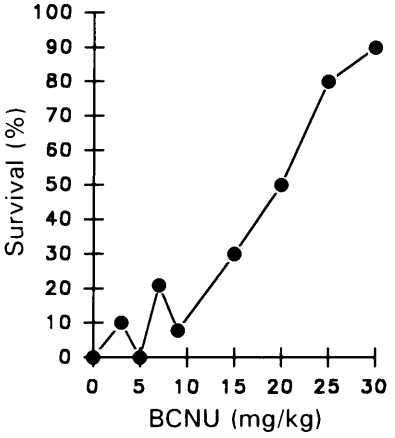

Figure 1. Dose of BCNU required to eradicate EL-4 tumor. Animals were injected ip with 1×10^5 EL-4 tumor cells, followed 4 days later with the indicated dose of BCNU. Survival was monitored for 60 days.

Effect of CS on the BCNU-Mediated Rejection of EL-4 Tumor. In order to investigate whether 30 mg/kg BCNU resulted in the total elimination of EL-4 tumor from the host, we employed CS, an inhibitor of T cell activation, to suppress an immune response to tumor. It was previously determined that 30 mg/kg/day CS for 21 days was sufficient to inhibit the BCNU and reovirus type 3–mediated rejection of EL-4 tumor (1). Figure 2 illustrates that the mean survival time of EL-4 tumor-bearing hosts was 18 days. Tumor-bearing mice that were treated with CS showed no significant deviation in the survival curve than tumor-bearing mice in the absence of CS. This demonstrated that CS had no apparent effect on tumor progression. Tumor-bearing mice treated with BCNU survived to high levels, with some experiments yielding 100% cure rates. In contrast, tumor-bearing mice that received both BCNU and CS typically yielded very low levels of survival. Non–tumor-bearing mice treated with both BCNU (30 mg/kg) and CS (30 mg/kg/day) showed no morbidity or mortality, demonstrating that the combined effect of both agents was not toxic to the animals. Analysis of the treatment groups showed that BCNU treatment slightly increased survival time compared with tumor controls, suggesting that BCNU may have reduced the tumor burden and thus allowing extended survival times. However, it was found that nearly all of the mice in the combined BCNU and CS group succumbed to tumor. These results indicated that a functional immune response, probably including an obligatory T cell–mediated component, was necessary to effect the total elimination of tumor burden from the host.

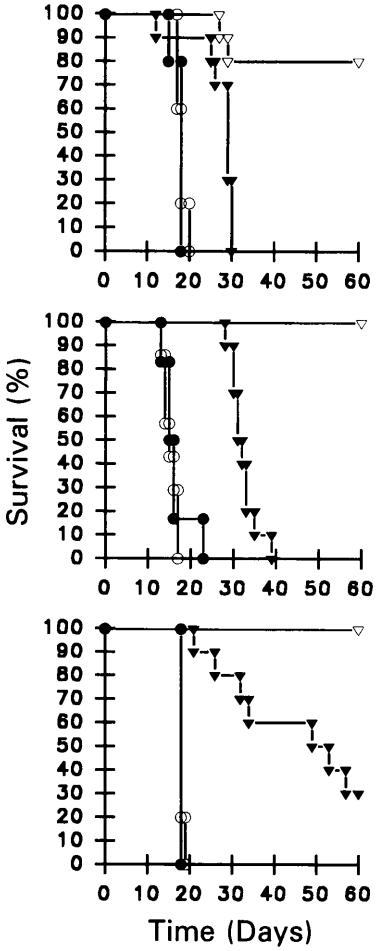

Figure 2. Effect of CS on EL-4 eradication by BCNU. All groups of animals were injected ip with 1×10^5 EL-4 tumor cells on Day 0. BCNU-treated animals received a single dose of 30 mg/kg at Day 4. CS-treated animals received a dose of 30 mg/kg/day for 21 days starting at Day −1. Survival was monitored for 60 days. Three separate experiments are shown. ○, tumor control; ●, CS; ▽, BCNU; ▼, CS + BCNU. The survival of BCNU groups varied significantly ($P < 0.05$) from CS + BCNU groups.

Effect of Delayed Administration of CS on BCNU-Mediated Tumor Therapy. Since CS produced a profound inhibitory effect on the rejection of tumor by BCNU, it was used to determine when the immune response had inflicted such damage on the tumor that recovery of tumor sufficient to produce host death could not occur. This was accomplished by staggering the start of CS administration at weekly intervals to BCNU-treated tumor-bearing animals. As shown in Figure 3, when CS administration was delayed so that it started at either 1, 2, or 3 weeks following tumor injection, no CS-mediated inhibition of the BCNU therapy took place. In contrast, when CS

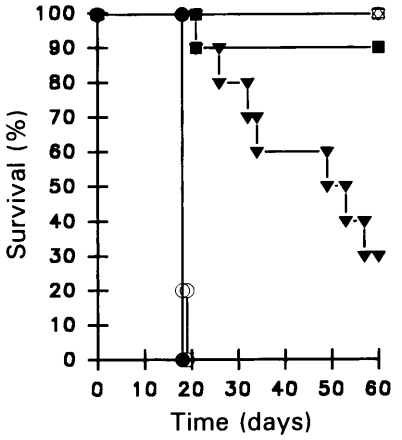

Figure 3. Effect of delayed CS administration. All groups of animals were injected ip with 1×10^5 EL-4 tumor cells on Day 0. All groups of animals, with the exception of tumor alone and tumor + CS groups, received a single injection of 30 mg/kg BCNU at Day 4. All CS-treated animals received a dose of 30 mg/kg/day for 21 days beginning on the indicated day. The tumor + CS group started receiving CS on Day −1. Survival was monitored for 60 days. ○, tumor control; ●, CS-Day −1; ▽, BCNU; ▼, BCNU + CS-Day −1; □, BCNU + CS-Day 7; ■, BCNU + CS-Day 14; △, BCNU + CS-Day 21. The survival of the BCNU + CS-Day −1 group varied significantly ($P < 0.05$) from the groups given CS at later intervals.

was administered starting at Day −1 relative to tumor injection, significant inhibition of the BCNU therapy occurred. These results suggest that the immune response to EL-4 tumor takes place early during the course of therapy and that by Day 7 after tumor injection significant damage to the tumor has resulted, such that inhibition by CS does not allow recovery of the tumor sufficient to cause the death of the host.

Determination of the Dose of BCNU Required to Eradicate L1210 Tumor. In order to determine whether the inhibitory effect of CS was specific to the EL-4 tumor system or whether it would have similar effects on other tumors, we examined the BCNU-mediated rejection of L1210 leukemia tumor cells. Like the EL-4 tumor system, we determined the effect of various doses of BCNU on L1210 tumor rejection *in vivo*. In our hands, the L1210 tumor was shown to be more sensitive to the effects of BCNU than EL-4, with the 50% survival level calculated from the curve to be approximately 11 mg/kg (Fig. 4). 100% survival of tumor-bearing mice was achieved at a dose of 20 mg/kg. As in the EL-4 tumor system, all 60-day survivors continued to live long term with no signs of morbidity. A dose of 15 mg/kg BCNU was chosen for further experiments as it provided a relatively high level of survival. A higher dose of BCNU was not used, because it might have killed all tumor cells, which in turn would have prevented investigation into the underlying immune response to the tumor.

Effect of CS on the BCNU-Mediated Rejection of L1210 Tumor. Figure 5 shows that, consistent with the EL-4 tumor system, CS exerted a strong inhibitory effect on the survival of combined BCNU- and CS-treated tumor-bearing mice. Tumor-bearing mice in the absence or presence of CS perished within 15–20 days after tumor injection, demonstrating that CS had no inhibitory effect on tumor progression. BCNU treatment of tumor-bearing animals provided a high level of survival. As observed using EL-4 tumor, BCNU treatment extended the survival time of the combined BCNU- and CS-treated tumor-bearing mice relative to mice that did not receive BCNU. However, nearly all of the mice perished within 50 days of tumor injection.

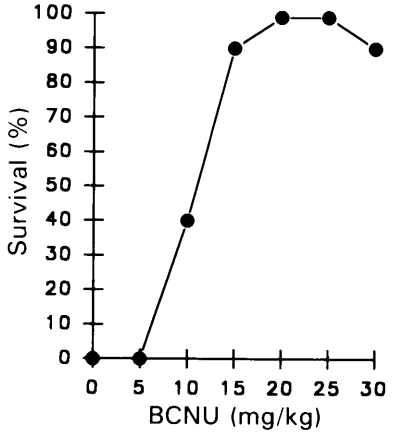

Figure 4. Dose of BCNU required to eradicate L1210 tumor. Animals were injected ip with 1×10^5 L1210 tumor cells, followed 4 days later with the indicated dose of BCNU. Survival was monitored for 60 days.

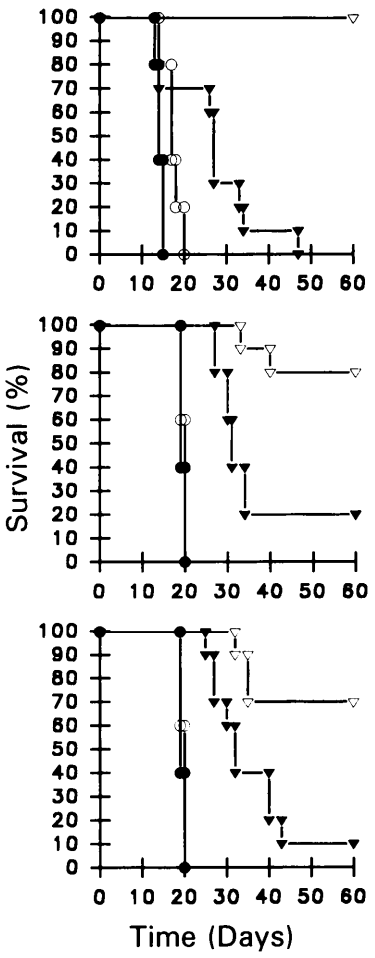

Figure 5. Effect of CS on L1210 eradication by BCNU. All groups of animals were injected ip with 1×10^5 L1210 tumor cells on Day 0. BCNU-treated animals received a single dose of 15 mg/kg at Day 4 and CS-treated animals received a dose of 30 mg/kg/day for 21 days starting at Day −1. Survival was monitored for 60 days. Three separate experiments are shown. ○, tumor control; ●, CS; ▽, BCNU; ▼, CS + BCNU. The survival of BCNU groups varied significantly ($P < 0.05$) from CS + BCNU groups.

Effect of CS on the Tumor Challenge of Cured Animals. Determining whether mice that had been cured of tumor by the BCNU therapy could withstand a lethal dose challenge of homologous tumor (i.e., the same tumor with which the mice were injected initially) was of interest. Also important was assessing whether CS could inhibit the successful rejection of homologous tumor by cured mice. Table I demonstrates that mice that had been cured of tumor using the BCNU therapy could successfully reject a lethal dose of homologous tumor with no signs of morbidity, indicating that an antitumor response sufficient to reject a lethal dose of tumor could be generated, but

TABLE I. Effect of CS on Tumor Challenge

Treatment[a]	Tumor	
	EL-4	L1210
Tumor alone	100	90 ± 10[b]
Tumor + CS	100	90 ± 10

[a] BCNU therapy–cured mice were rechallenged with 1×10^5 homologous EL-4 or L1210 tumor cells in the presence or absence of 30 mg/kg/day CS for 21 days starting at Day −1. Survival was monitored for 30 days.
[b] The data represents the mean survival (%) ± SEM of three experiments.

without the requirement for BCNU. CS could not inhibit this antitumor response in cured mice, consistent with its diminished ability to inhibit secondary immune responses compared with primary immune responses (8, 9).

Discussion

In the interest of discerning whether the BCNU-mediated rejection of tumor required an obligatory immune response component, we undertook the present series of investigations. Other studies have examined tumor-specific immune cell populations in mice that have been cured of tumor using BCNU. However, it is technically difficult to study tumor-specific immune cell populations during the active elimination of tumor, when the immune cell populations may be relatively small. Therefore, we chose to pharmacologically inhibit the immune response using CS, because CS could be present throughout the course of the therapy and because the *in vivo* physiologic environment would be maintained. We demonstrated that CS could suppress the BCNU-mediated rejection of both EL-4 and L1210 tumor cell lines.

BCNU is a highly lipid soluble compound that has been demonstrated in humans to have cytotoxic activity against a variety of tumors such as malignant melanoma, gastrointestinal carcinomas, certain brain cancers, multiple myeloma, and Hodgkin's and non-Hodgkin's lymphomas (2). Chemical breakdown of BCNU yields a chloroethyldiazohydroxide and an isocyanate group, both highly reactive metabolites (11). The isocyanate group reacts with amine groups in a carbamoylation reaction and is thought to alter the maturation of RNA, deplete glutathione levels, and restrict DNA repair mechanisms. The importance of the carbamoylation reaction in the killing of tumor cells is questionable, given that other nitrosoureas that lack this activity still retain potent antitumor activity. As the chloroethyldiazohydroxide decays, it generates reactive chloroethyl carbonium ions that form DNA adducts with all four bases. A more significant activity of BCNU is the formation of DNA interstrand cross-links which are closely correlated with cell cytotoxicity (12).

The L1210 tumor cell line is derived from a lymphocytic mouse leukemia, whereas EL-4 is a murine lymphoma cell line. We demonstrated that L1210 tumor destruction was more sensitive to lower BCNU concentrations than EL-4. These findings may be due to the intrinsic sensitivity of the cell lines to BCNU or may involve differences in immunogenicity of the two cell lines. L1210 is generally thought to be more immunogenic than EL-4 (13) and therefore may require less tumor burden to be destroyed by BCNU in order to achieve survival levels similar to EL-4.

The ability of cured animals to resist homologous tumor rechallenge suggests that the BCNU-mediated tumor killing allowed the tumor cells to persist for a time period sufficient for tumor cell antigens to stimulate vigorously an immune response. Alternatively, BCNU could influence the immunogenicity of the tumor cells to provoke a more robust immune response than untreated tumor cells. Regardless of the mechanism, it is clear that BCNU can render the cured host resistant to further tumor challenge. It is noteworthy that the cured mice eliminated tumor in the absence of BCNU, indicating that a significant expansion in the tumor-killing capability of the immune system occurred.

CS has been reported to have a cytostatic effect on numerous malignant cell lines *in vitro* (14), mainly T cell tumor lines. EL-4 tumor cells are susceptible to CS-mediated DNA synthesis suppression as a result of ornithine decarboxylase inhibition (15). However, the findings that tumors can be inhibited *in vitro* should be cautiously interpreted with respect to the potential for *in vivo* inhibition. CS exerted an antiproliferative effect on B16F10 melanoma cells *in vitro*, but enhanced the implantation of tumor in the liver of syngeneic mice (16). The ability of CS to influence *in vivo* L1210 or EL-4 (Fig. 2 and 5) growth was evaluated and found to have no effect on the survival time of the mice compared with untreated mice, though some tumor cell reduction may have taken place (17). In our hands, 30 mg/kg/day CS produced no mortality or apparent morbidity in the mice. Indeed, the immunosuppressive effect of CS apparently allowed the residual viable EL-4 or L1210 tumor cells, following BCNU treatment, to emerge and eventually to kill the mice.

Evidence supporting the need for an intact immune response in the successful chemotherapeutic eradication of tumor comes from various sources. Mathe *et al.* found that immunosuppression of mice following treatment with antithymocyte serum, thus depleting the mice of T lymphocytes, significantly reduced the ability of cyclophosphamide to cure mice of L1210 leukemia cells (18). In addition, treatment of LSA thymic lymphoma–bearing syngeneic mice with BCNU was found to produce over 90% survival. However, the same treatment failed to cure mice of tumor

burden when the mice were first irradiated or nude tumor-bearing mice, essentially devoid of T cell–mediated immunity, were used (19). Finally, streptozotocin, a nitrosourea that has tumoricidal properties similar to BCNU, was found to be unable to cure LSA tumor–bearing mice. This was presumably due to the inability of streptozotocin to eliminate tumor-specific suppressor cells, a function that BCNU retains (20).

These studies suggest that in immunocompromised patients undergoing chemotherapy, either directly through the use of immunosuppressive drugs or indirectly through chemotherapy-induced immunosuppression, important host antitumor responses may be compromised.

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