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Immunomodulation and Enhancement of Antitumor Activity by Co-administration of 1,3-Bis(2-chloroethyl)-1-nitrosourea and Thymidine

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ABSTRACT. The antitumor activity of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been shown previously to be enhanced markedly by the co-administration of pyrimidine deoxyribonucleosides (Lin and Prusoff, *Cancer Res* **47:** 394–397, 1987). In the present study, we examined the cellular mechanisms underlying the augmentation effect of thymidine, one of the pyrimidine deoxyribonucleosides. It was found that thymidine did not increase the cytotoxicity of BCNU for B16/F10 melanoma cells *in vitro*. Instead, thymidine appeared to produce modulatory actions on the immune system of the tumor-bearing mice. More than 40% of the BCNU/thymidine-cured mice specifically rejected secondary rechallenge with the B16/F10 tumor. Furthermore, these cured mice developed extensive depigmentation of their natural black hair, suggesting immune reactions to normal melanocytes. When spleen cells from normal mice were treated with BCNU alone, their response to T-cell mitogen phytohemagglutinin was suppressed markedly. This suppression was ablated by co-administration of BCNU with thymidine. Such BCNU/thymidine treatment also augmented the activity of tumor-specific cytotoxic T-cells in tumor-bearing mice. Taken together, these results suggest that the enhanced antitumor activity of combined BCNU and thymidine may result from the action of thymidine on the immune effector mechanisms, which facilitate the development of antitumor immune responses in the presence of immunosuppression induced by BCNU. BIOCHEM PHARMACOL 53;5:705–713, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); thymidine; melanoma; immunomodulation; antitumor immune response

The nitrosoureas, a group of lipophilic chemicals with potent alkylating ability, are known to be effective chemotherapeutic agents against a variety of malignancies such as brain tumors and melanoma [1]. Among various nitrosoureas, BCNU† is one of the most potent agents against solid tumors [2]. However, there are problems associated with the clinical use of high-dose BCNU, which include delayed myelosuppression [3] and occurrence of pulmonary toxicity [4]. One approach to enhancing the effectiveness of BCNU treatment without increasing its dosage level and toxicity is the combined use with other modulatory drugs. It is well-known that activity of many antitumor agents can be modulated by thymidine or other pyrimidine deoxyribonucleosides. For example, administration of thymidine has been found to enhance the cytotoxicity of 5-FU and methotrexate in cultured human colon carcinoma [5], and 6-aminonicotinamide potentiates the antileukemic effect of BCNU in mice bearing L1210 leukemia [6].

Lin and Prusoff [7] reported that co-administration of a single dose of pyrimidine deoxyribonucleosides with BCNU to mice bearing B16/F10 melanoma results in a significant increase in the number of long-term survivors (60–120 days). However, the effects of thymidine on the cytotoxicity of various chemotherapeutic agents alone cannot explain the augmentation of antitumor activity. In fact, an *in vitro* study has shown that thymidine decreases the cytotoxicity of the synthetic thymidine nitrosourea analogue 3′-CTNU against L1210 cells [8]. In the same study, thymidine was also found to have no effect on the cytotoxic activity of BCNU to L1210 cells in culture. In the present work, we confirmed the finding that thymidine has no effect on the cytotoxic activity of BCNU in a culture system of melanoma cells.

The use of a tumoricidal drug such as BCNU in cancer therapy has not ensured long-term survival or complete cure because of the escape of tumor cells from the drug action. Thus, in addition to a drug-mediated reduction of tumor burden, it would be beneficial to have the immune system of the host operating simultaneously to ensure that

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† *Abbreviations:* BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; PHA, phytohemagglutinin; 3′-CTNU, 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine; MEMH, minimum essential medium with Hanks' salt; FBS, fetal bovine serum; RPMI 1640, Roswell Park Memorial Institute 1640 medium; Con A, concanavalin A; SR, spontaneous release; HBSS, Hanks' balanced salt solution; DTH, delayed-type hypersensitivity; ADPRP, poly(ADP-ribose) polymerase; and 5-FU, 5-fluorouracil.

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no tumor cells survive. However, chemotherapeutic agents are generally considered to exert their antitumor effects through antiproliferative and cytotoxic actions. This very same mechanism often induces bone marrow toxicity and immunosuppression. We have examined the biomodulation activity of thymidine when co-administered with BCNU on the host antitumor immune response to B16/F10 melanoma in C57BL/6 mice. The results of the present study suggest that the augmentation by thymidines of the antitumor activity of BCNU may be accounted for by its modulation of immune effector mechanisms, either through an inhibitory or a compensatory action on the immunosuppressive effects of BCNU.

MATERIALS AND METHODS

Animals

Adult, female C57BL/6 (H-2^b) mice, obtained from NIH (Frederick, MD), were kept in the animal facility of Yale University and given food and water *ad lib*. They were used when they were 10–12 weeks old. For any one experiment, the animals were distributed into groups of eight to twenty mice of similar weight and maintained throughout the course of the experiment. The mice were weighed daily during the experiment, and the percentage change in body weight and lethality were used as a measure of drug toxicity. Autopsies were performed on the mice that died during the experiment to determine whether the cause of death was due to drug toxicity or tumor overgrowth. The evaluation of the therapeutic effect of the drug on tumor-bearing mice was based on the increase in median survival time and the number of long-term survivors (≥60 days) achieved by different treatments.

Tumors

The B16/F10 melanoma, syngeneic to C57BL/6 mice, was obtained from Dr. I. J. Fiddler (MD Anderson Cancer Institute, Houston, TX). Cells were propagated as a solid tumor in C57BL/6 mice. Transplantation was carried out by removing tumors from donor mice bearing 14-day subcutaneous tumor growth. The tissue was fragmented to make a well-dispersed cell suspension and diluted with MEMH. Tumor cells (10^5 cells in 0.2 mL PBS, pH 7.2) were injected i.p. into each recipient animals.

Cell Lines

B16/F10 cells were maintained *in vitro* in MEMH supplemented with 5% heat-inactivated FBS (Gibco Laboratories, Grand Island, NY) and antibiotics (MEMH-5). EL-4, a chemically induced leukemia also syngeneic to C57BL/6 mice, was obtained from Dr. C. Janeway (Yale University) and maintained in RPMI 1640 supplemented with 10% FBS (RPMI-10; Gibco Laboratories). EL-4 cells are used as LAK-sensitive target cells and heterologous syngeneic tumor challenge. YAC-1, a Moloney leukemia of A/Sn origin, was obtained from Dr. C. Janeway and maintained in RPMI-10 culture medium. YAC-1 cells were used as a natural killer cell-sensitive target.

Drug Treatment

BCNU formulated for clinical use as Carmustine (Bristol–Meyer Squibb, U.S.A.) was weighted and dissolved immediately before use and protected from light. Thymidine of 99–100% purity was acquired from Sigma (St. Louis, MO). BCNU was dissolved in ethanol and diluted further with PBS, whereas thymidine was dissolved directly in warm PBS. For *in vivo* drug treatments, BCNU was injected i.p. at a concentration of 20 mg/kg body weight, 24 hr after tumor cell inoculation. Thymidine was also given i.p. at a concentration of 2 g/kg body weight simultaneously with BCNU. Control animals, which were injected with equal volumes of drug vehicle (PBS), were included in each experiment. For *in vitro* drug treatments, tumor cells were incubated in 10 μM BCNU with or without 100 μM thymidine at 37° for various time intervals.

Mitogenic Stimulation of Spleen Cells

Spleen cells were removed aseptically from mice. Single cell suspensions were made, and erythrocytes were lysed in ammonium chloride/potassium lysing buffer. Cell proliferation to mitogens was measured using a [^{3}H]thymidine incorporation assay. Spleen cells from normal mice following drug treatments were plated in triplicate at a concentration of 2×10^5 cells/well in 96-well round-bottomed plates in 200 μL of culture medium with the mitogens Con A or PHA at final concentrations of 2 or 10 μg/mL, respectively. Cultures were incubated at 37° for 48 hr. Cells were pulsed with 2 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) 18 hr prior to cell harvesting. Cells were harvested onto glass fiber filter paper and counted with scintillation fluid in a liquid scintillation counter (Beckman, CA). The results are expressed as mean cpm ± SEM of triplicate cultures.

Cytotoxicity Assay

Cell-mediated cytotoxicity was assayed by the chromium release method. Target cells (5×10^6 cells) were incubated with 100 μCi of [^{51}Cr]sodium chromate for 1 hr at 37° and 10^4 ^{51}Cr-labeled cells were added to each well. Target cells for the cytotoxicity assay were B16/F10, EL-4, and YAC-1. Effector cells (splenocytes) from tumor-bearing mice were mixed with target cells at the starting ratio of 50:1. The U-bottom 96-well microtiter plates were spun briefly and then incubated at 37° for 8 hr before the supernatant was harvested for gamma-counting. The maximum release of [^{51}Cr] was from 1 N HCl-treated cells. SR, which was consistently below 25% of maximum releasable counts, was that released by target cells incubated in medium alone.

Percent specific [^{51}Cr] release was then calculated according to the formula:

$$\frac{\text{Experimental release (cpm)} - \text{SR (cpm)}}{\text{Maximum release (cpm)} - \text{SR (cpm)}} \times 100$$

Variations in the percentage of [^{51}Cr] release between individual samples of each triplicate rarely exceeded 5%. Each experiment was performed two or three times.

Delayed-Type Hypersensitivity Reaction

B16/F10 tumor cells (10^6) were first irradiated at 10,000 rad, followed by resuspension in PBS, and then inoculated into the right footpads of the mice. Left footpads were injected only with PBS as controls. Footpad swelling was measured using a dial gauge micrometer (Mitutoyo) 24 hr later and expressed in units ± SEM (1 unit = 10^{-2} mm).

RESULTS

Therapeutic Effect of BCNU and BCNU/ Thymidine on Mice Bearing B16/F10 Melanoma

As a basis for studying the cellular mechanisms involved in the combined treatment with BCNU and thymidine, we first examined the antitumor effect of BCNU with or without thymidine in C57BL/6 mice bearing B16/F10 melanoma. A single i.p. treatment of BCNU (20 mg/kg) with or without a single i.p. injection of thymidine (2 g/kg) was administered 1 day after i.p. injection of 10^5 viable tumor cells. As shown in Table 1, the control mice (PBS alone) and mice that received thymidine alone died on an average of 14.0 and 14.5 days after tumor implantation, respectively. The group of mice that received just BCNU died after an average of 21.9 days with only 1 out of 20 mice surviving beyond 60 days. When thymidine was co-administered with BCNU, the average life span was increased to 31.4 days, with 5 out of 19 mice surviving more than 60 days. The surviving mice also regained their body weight. Thus, co-administration of thymidine with BCNU produced marked augmentation of antitumor activity, consistent with a previous finding [7].

Tumoricidal Effect of BCNU/ Thymidine on B16/F10 Melanoma Cells In Vitro

To define the tumoricidal effect of combined BCNU/thymidine treatment, independent of immune effector mechanisms, the efficiency of tumor cell killing *in vitro* by BCNU, thymidine, or BCNU/thymidine was examined. B16/F10 cells were plated in 24-well tissue culture plates in MEMH-5 complete medium (2×10^5 cells/well). Drugs were added 24 hr later, and the incubation was continued for another 20, 44, and 70 hr. Treated tumor cells were removed with 0.2% EDTA in HBSS without Ca^{2+} and Mg^{2+} and washed and resuspended in MEMH-5 complete medium. Viable cells were counted by the trypan blue dye exclusion test. The number of viable cells in drug-treated cultures was normalized by that of the parallel control cultures (without drug treatment) to assay tumor cell survival after various drug treatments. As shown in Fig. 1, treatment with thymidine (100 μM) alone had no effect on tumor cell survival ($P = 0.68$, t-test). Co-administration of thymidine (100 μM) with BCNU (10 μM) resulted in reduced tumor survival similar to that produced by BCNU (10 μM) alone at 20 and 44 hr. However, after 70 hr the BCNU/thymidine-treated cultures had higher cell survival than that of BCNU-treated cultures (72 ± 5.4 and 51 ± 9.2%, respectively, $P < 0.01$, t-test), suggesting reduced cytotoxicity of the BCNU/thymidine treatment. Thus, co-administration of thymidine did not enhance the tumoridical activity of BCNU *in vitro*, but actually decreased this activity after prolonged treatment. A similar study [8] with L1210 cells *in vitro* found no effect of thymidine on BCNU cytotoxicity even after 3 days of incubation.

Resistance of Cured Mice to Tumor Challenge

Long-term survivors of BCNU/thymidine treatment could be associated with the development of host resistance to

TABLE 1. Survival of mice bearing the B16/F10 melanoma after BCNU treatment with or without thymidine

Drug*	Average survival time† (days ± SEM)	Long-term survivors‡	T/C × 100§
Control (PBS)	14.0 ± 0.5	0/19	100
Thymidine (2 g/kg)	14.5 ± 0.2	0/20	103
BCNU (20 mg/kg)	21.9 ± 0.9	1/20	156
BCNU (20 mg/kg)/ Thymidine (2 g/kg)	31.4 ± 1.4	5/19	224

* Drugs were administered by a single i.p. injection, 24 hr after implantation of B16/F10 melanoma cells.

† Average survival time includes only those mice that died prior to day 60 (mean ± SEM, N = 19 or 20).

‡ Value represents the number of mice that survived beyond 60 days divided by the total number of mice treated.

§ T/C × 100 represents the ratio of the average survival time of the treated group to that of the control group × 100 for the animals that died.

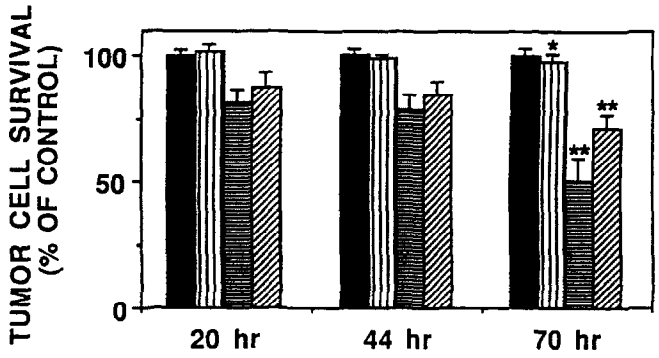

FIG. 1. Tumoricidal effects of BCNU, thymidine, and BCNU/thymidine on cultured B16/F10 melanoma cells (2×10^5). Columns: PBS control cultures (■) and cultures treated with 100 μM thymidine (▥), 10 μM BCNU (▤), or 10 μM BCNU and 100 μM thymidine (▨) for 20, 44, or 70 hr. In each experiment, triplicate cultures were counted and averaged for each time point, and the cell count was normalized against the values observed for the control cultures in the same experiment. Each bar represents the mean ± SEM from three separate experiments. Key: (*) $P = 0.68$ vs control and (**) $P < 0.01$ BCNU-treated vs BCNU/thymidine-treated.

tumors, characteristic of antitumor immunity. This possibility was tested by challenging mice that had survived the initial B16/F10 melanoma inoculum after BCNU/thymidine treatment with i.p. injection of live B16/F10 cells, beginning at day 60 after the initial tumor implantation. The first challenge was an inoculation of 10^2 B16/F10 cells, followed by inoculations of 10^3 cells at 3 weeks, 10^4 cells at 6 weeks, and 10^5 cells at 9 weeks. Figure 2 shows the percent of surviving mice following tumor challenge in control mice and mice cured with BCNU/thymidine. More than 40% of the cured mice survived the tumor challenge, while all control (naive) mice inoculated with the same protocol died within 5 weeks of tumor challenge. This resistance to tumor challenge is tumor-specific, since the cured mice died at similar rates as the control mice after inoculation with heterologous syngeneic tumor EL-4 cells.

Depigmentation in the BCNU/Thymidine-Cured Mice

The above study showed that treatment with BCNU/thymidine resulted in an improved survival of tumor-bearing mice. Interestingly, we noted that the surviving animals developed depigmentation of their cranial-dorsal black hair 30 days after receiving the treatment, and the depigmentation extended further to the caudal-dorsal area at 80 days after the treatment. Representative BCNU/thymidine-cured mice and age-matched control mice are shown in Fig. 3A. The depigmentation continued to progress to the entire body after rechallenge with live B16/F10 cells (Fig. 3B). Since localized depigmentation (vitiligo) has been shown to result from immune response to melanocytes [9], the present finding supports the notion that BCNU/thymidine treatment induced immunogenic changes in the tumor-bearing mice, leading to an autologous reaction to normal melanocytes.

Elicitation of a DTH Response In Vivo by BCNU/Thymidine-Cured Mice but Not by Normal or Tumor-Bearing Mice

Since earlier experiments demonstrated that BCNU/thymidine-cured mice rejected a secondary challenge with the homologous tumor but not the heterologous tumor, further studies were carried out to investigate whether the mechanism of protection involved a tumor-specific, DTH response. Normal, tumor-bearing, and BCNU/thymidine-cured mice were challenged in the footpad with irradiated (10,000 rad) B16/F10 tumor cells, and the footpad swelling was measured 24 hr later (Fig. 4). Both normal and tumor-bearing mice failed to elicit B16/F10-specific DTH responses. In contrast, BCNU/thymidine-cured mice elicited a strong DTH response to B16/F10 tumor cells. The response was tumor specific, since injection of the heterologous syngeneic tumor EL-4 elicited no DTH response. These data suggest that BCNU/thymidine-cured mice have developed immune reactions specifically against the weakly immunogenic B16/F10 cells.

Effect of Treatment of Normal Mice with BCNU, Thymidine, and BCNU/Thymidine on the Mitogenic Response of Spleen Cell

Whether BCNU, thymidine, or BCNU/thymidine had any immunosuppressive effect was examined further by assaying the mitogenic response of the spleen cells from normal mice. On day 12 after the drug treatment, spleen cells were harvested and stimulated with T-cell mitogens. As shown in Table 2, BCNU suppressed the proliferation of spleen cells in the presence of the mitogens Con A and PHA at 12 days after the drug treatment. On the contrary, administration of thymidine alone did not suppress the mitogen response to either Con A or PHA. When thymidine was administered concurrently with BCNU, it greatly reduced the suppression of PHA-response induced by BCNU to that of the control level (with no drug treatment), but the suppression of Con A response was not changed. Taken together, these data suggest that BCNU, when used in combination with thymidine, induces less immunosuppression assessed by *in vitro* PHA mitogen response of spleen cells.

Effect of BCNU/Thymidine Treatment on Immune Effector Cells (Natural Killer Cells, Lymphokine-Activated Killer Cells, and Tumor-Specific Killer Cells)

Although thymidine prevented the immunosuppression in the *in vitro* T-cell response to PHA, it is not clear how these data could reflect on the antitumor immunity *in vivo*. The complexity of antitumor immune response demands a finer dissection of the specific effect of BCNU/thymidine on various cell types in the immune system. Thus, we further

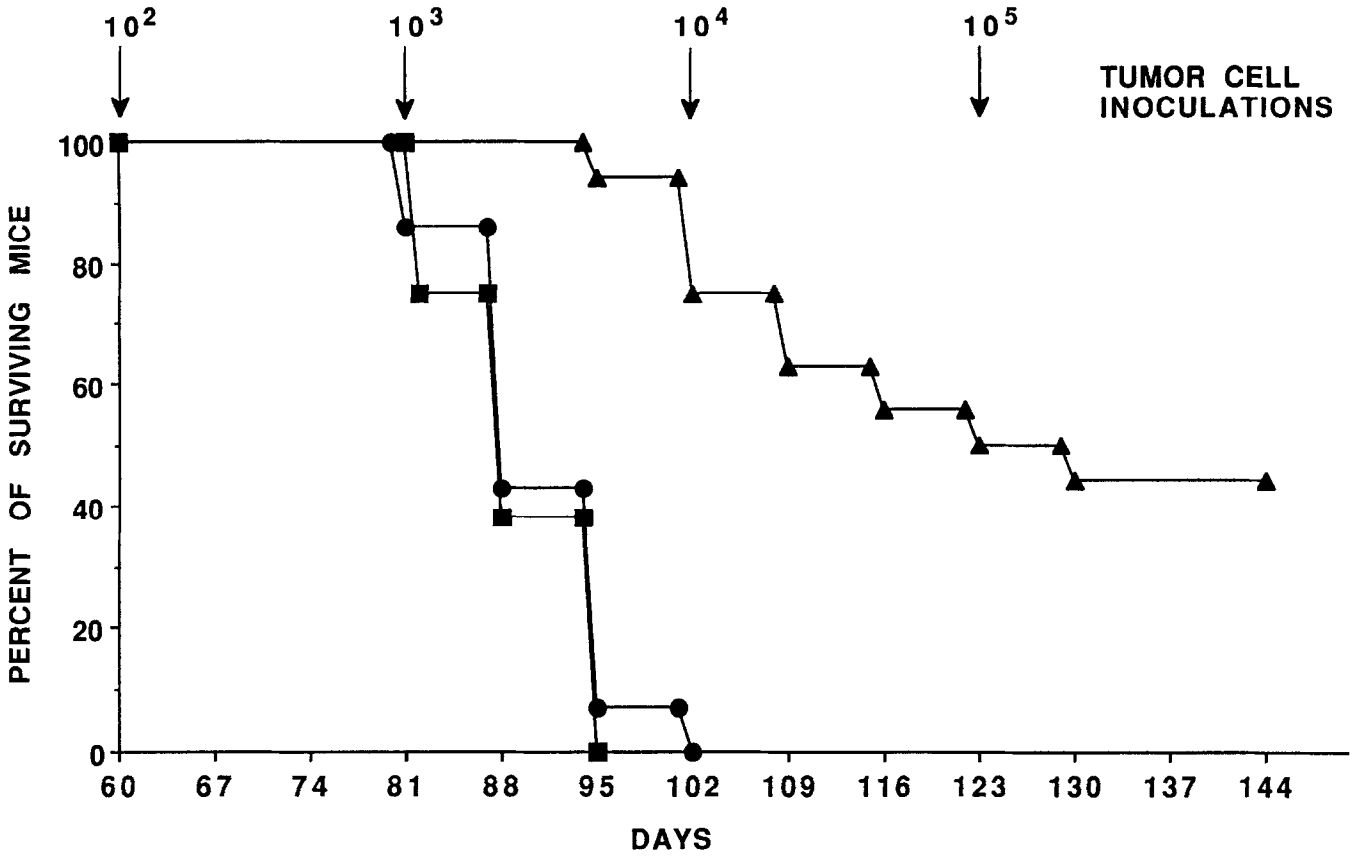

FIG. 2. Survival of mice cured by the co-administration of BCNU (20 mg/kg) plus thymidine (2 g/kg), after rechallenge with viable B16/F10 cells. Control mice (14 mice/group) received no drug treatment and were challenged only with B16/F10 cells (●). BCNU/thymidine-cured mice were challenged with B16/F10 cells (16 mice/group, ▲) or EL-4 cells (8 mice/group, ■). Tumor cells were injected once every 3 weeks with escalating dosages (marked by arrows), beginning at day 60 after initial tumor implantation.

examined various cytotoxic functions of the spleen cells of tumor-bearing mice treated with BCNU or BCNU/thymidine, as a functional assay for various effector cell populations. Spleen cells, prepared from various treatment groups at day 12 post-treatment, were used as effector cells in the [^{51}Cr]-release assay, and [^{51}Cr]-labeled YAC-1, EL-4, and B16/F10 melanoma cells were used as NK, LAK, and tumor-specific CTL target cells, respectively. The effector to target cell ratio was 50:1 and 25:1. As shown in Table 3, the low-level of LAK cell activity (EL-4 as target cells) was not altered by various treatments. However, the low-level NK cell activity (YAC-1 as target cells) was enhanced minimally by co-administration of thymidine with BCNU. Spleen cells of tumor-bearing mice with or without the BCNU treatment were devoid of tumor-specific CTL activity up to 10 days after the drug treatment (data not shown). The activity of tumor-specific CTL (B16/F10 as target cells) detected at 12 days after BCNU treatment was enhanced significantly by the co-administration of thymidine. Further increase of the tumor-specific CTL activity was observed in the spleen cells of mice surviving 80 days after BCNU/thymidine treatment. We therefore concluded that BCNU or BCNU/thymidine treatment did not change the activity of NK cells in tumor-bearing mice, but that tumor-specific CTL activity was enhanced in mice treated with BCNU/thymidine. These CTLs could play an active role in killing tumor cells and the development of tumor-specific immunity.

DISCUSSION

BCNU is an effective antitumor agent against a number of experimental tumors [10–12]. When used as a single agent clinically, it is one of the most active chemotherapeutic agents for the treatment of metastatic melanoma, with response rates between 10 and 18% at dosage levels below the maximal tolerated dose [13]. Despite the serious dose-limiting toxicity of delayed suppression of hematopoiesis, higher doses of BCNU have been used in the treatment of advanced melanoma, with the help of autologous bone marrow rescue or multiple blood transfusion [14, 15]. The high dose therapy had increased response rates of 22–27%, but toxicity was severe and the responses were associated with unacceptable fatality rates. Thus, more recent treatments of metastatic melanoma involve the use of several other chemotherapeutic agents in combination with a lower dosage of BCNU. Another potential approach to increase the efficacy of BCNU treatment is the use of bio-

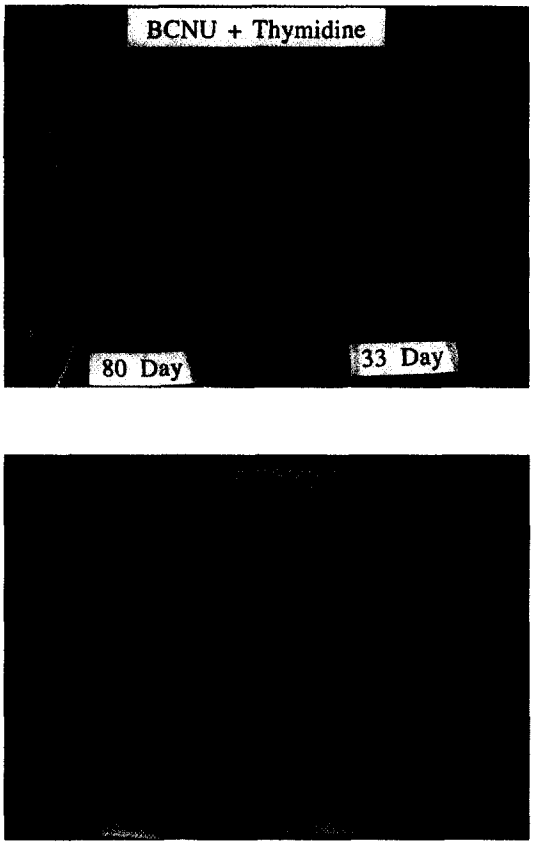

FIG. 3. Development of depigmentation in tumor-bearing C57BL/6 mice following the administration of BCNU (20 mg/kg) plus thymidine (2 g/kg). (A) Mice with depigmentation (on the left of each set) at 33 days and 80 days after tumor implantation and administration of BCNU/thymidine; age matched control mice (no tumor and no treatment) are shown on the right of each set. (B) Depigmentation in BCNU/thymidine-cured mice who survived after the rechallenge with B16/F10 tumor cells, 260 days after the initial tumor implantation and administration of BCNU/thymidine. Age-matched control animal (no tumor and no drug treatment) is shown on the right.

modulators, e.g. 6-aminonicotinamide, since such combination treatments have been shown to be effective in animals studies [6]. In the present study, we have examined the potential usefulness and immunomodulatory mechanisms of thymidine when used in combination with BCNU. Thymidine was administered concurrently with BCNU. We have not investigated the effect of variation of the administration of BCNU and thymidine.

Thymidine has been used extensively to synchronize mammalian cell growth *in vitro* [16, 17]. Results of clinical studies have shown that high-dose continuous infusion of thymidine alone has antitumor activity in patients with T-cell leukemia or cutaneous T-cell lymphoma, although it is ineffective against solid tumors or B-cell disorders [18, 19]. This suggests that thymidine may be a candidate as an immune modulator when used with other chemotherapy agents. In previous studies, co-administration of a single dose of thymidine with either BCNU or 3′-CTNU to L1210-bearing mice has been shown to enhance significantly the antitumor activity of these two nitrosoureas [7, 20]. Since the increased effectiveness was not obtained at the expense of increased toxicity, the combination of thymidine and BCNU has obvious clinical potential. In the present study, we have extended these earlier findings by demonstrating the effective antitumor activity of BCNU/thymidine in treating mice bearing melanoma, a non-lymphoid solid tumor. More importantly, we have shown that, in addition to an increased average duration of survival, there was a significant increase in the number of long-term survivors ($\geq$60 days).

The mechanism underlying the enhanced effectiveness of BCNU/thymidine treatment in animal models is largely unknown. The most obvious possibility is an increased tumoricidal activity of BCNU or 3′-CTNU by co-administration of thymidine. It was reported that thymidine was an inhibitor of ADPRP, an enzyme activated in response to nitrosourea-induced DNA damage. However, the observed potentiation of 3′-CTNU activity by thymidine does not appear to result from such a mechanism [21]. Moreover, the inhibitory action of 3′-CTNU but not of BCNU on L1210 cell proliferation *in vitro* was actually reduced by the addition of thymidine [8]. This difference is due to the inhibition of the cytotoxicity of 3′-aminothymidine, which is a hydrolytic product of 3′-CTNU. In the present study, thymidine did not alter the cytotoxicity of BCNU against B16/F10 cells *in vitro*. Thus, the enhanced antitumor activity of BCNU/thymidine is unlikely to be accounted for by an increased tumoricidal activity. Therefore, other mechanisms, e.g. enhancement of antitumor immune responses by thymidine, may be involved.

In animal studies, BCNU has been found to be particularly potent in treating lymphoid tumors, e.g. a syngeneic spontaneous thymic lymphoma LSA and Moloney-virus-induced lymphoma YC8 [11, 12]. The effectiveness of BCNU treatment in mice bearing these tumors requires the contribution of T-cell-dependent antitumor immunity and that cured mice become resistant to subsequent rechallenge with the homologous tumor cells [11, 12]. In addition to a drug-mediated direct tumoricidal activity, BCNU appears to kill preferentially T-suppressor cells, which may allow the host to mount an effective antitumor response [22]. In the present study using a single injection, treatment with BCNU alone in mice bearing B16/F10 melanoma rarely led to long-term survival (1 in 20), while combination treatment of BCNU/thymidine cured a significantly higher number of mice (5 in 19). Furthermore, over 40% of the BCNU/thymidine-cured mice were resistant to subsequent rechallenges with B16/F10 tumor cells but not with a different tumor such as EL-4, which is also syngeneic to the C57BL/6 mice. These findings suggest that BCNU alone produces rather limited antitumor activity in this non-lymphoid tumor model and that the increased efficacy of the combined BCNU/thymidine treatment appears to re-

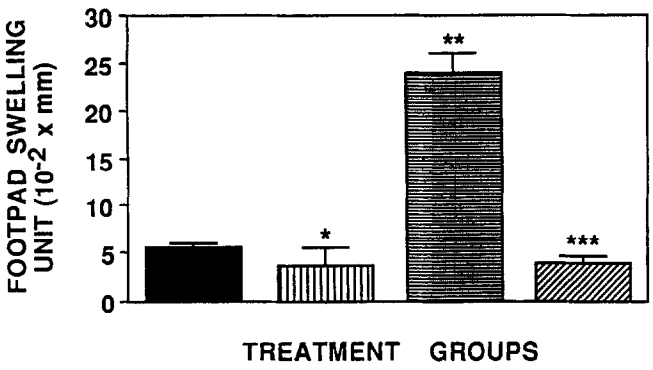

FIG. 4. DTH reaction in normal, B16/F10 tumor-bearing, and BCNU/thymidine-cured mice. Control mice (■), tumor-bearing mice (▥) and BCNU/thymidine-cured mice (▤) were injected in the footpads with 10^6 irradiated B16/F10 cells, and the BCNU/thymidine-cured mice were also injected with 10^6 EL-4 tumor cells (▨). The footpad swelling was measured 24 hr later as an assay of DTH. The swelling was significantly higher in BCNU/thymidine-cured mice injected with B16/F10 (* $P = 0.34$, ** $P = 0.0003$, *** $P = 0.053$ vs control). Data are means ± SEM from three separate experiments.

sult from a synergistic action of the drugs on the immune system. Although thymidine has a very short half-life in blood, due to first pass conversion to thymine by the liver, it diffuses rapidly into cells where it is protected from degradation by phosphorylation to the mono-, di- and triphosphate derivatives.

Involvement of the immune system is further suggested by depigmentation of the cured mice after BCNU/thymidine treatment. The appearance of white patches on the skin (vitiligo) is seen in a number of melanoma patients, especially in patients with metastatic melanoma who received sequential chemo-immunotherapy and underwent partial destruction of their melanocytes [23]. The autologous anti-tyrosinase CTL clones have been implicated for both the tumor rejection and destruction of normal melanocytes [24]. Furthermore, the autoantibodies against melanocytes have been detected with a high frequency in patients with vitiligo [25]. Although it remains unclear whether these autoantibodies in melanoma patients are produced in response to the therapy, several groups have reported that the presence of vitiligo is associated with improved survival in patients with metastatic melanoma even in the absence of systemic therapy [26, 27]. These findings are consistent with the hypothesis that an autoimmune response against normal melanocytes may be related to the immune response involved in rejecting melanoma cells. In our study, we observed that depigmentation developed at 30 days following BCNU/thymidine treatment. This process was even more prominent after the BCNU/thymidine-cured mice were challenged again with B16/F10 melanoma cells. These findings suggest that autoimmune responses against normal melanocytes, which share antigenic components with B16/F10 tumor cells, are induced in the BCNU/thymidine-cured mice. The autoreactive effector cells and/or autoantibodies may be essential in eliminating the tumor cells escaping from the cytocidal action of BCNU and in resisting the subsequent tumor cell challenge. Preliminary data (Poo, W-j and Halaban R, unpublished findings) indeed show that such antibodies against murine melanocytes can be readily detected in the serum of BCNU/thymidine-cured mice but not in control or tumor-bearing untreated mice.

Immunomodulators such as levamisole have been used successfully in combination therapy with 5-FU in the adjuvant therapy for patients with stage III colorectal cancer following surgical resection. This combination therapy has reduced tumor recurrence significantly and improved overall survival [28]. The mechanism of action of levamisole, alone or in combination with 5-FU, is unknown. Positive interaction of levamisole and 5-FU has been attributed to the restorative effects on depressed cell-mediated immune responses, although no extensive immunologic analysis was provided in the clinical studies [29]. Under the conditions used in our experiments, BCNU is toxic for all subsets of splenocytes in normal mice even at day 12 following drug treatment. T-cell responses to Con A and PHA were suppressed markedly. In contrast, thymidine alone did not exhibit any immunosuppressive effect in normal mice.

TABLE 2. Effect of BCNU and thymidine on T-lymphocyte subsets in normal mice*

Drug†	Con A-induced mitogenesis‡ (cpm)	PHA-induced mitogenesis‡ (cpm)
Control	137,600 ± 1,900	30,400 ± 800
Thymidine (2 g/kg)	133,900 ± 1,500	34,400 ± 1,000
BCNU (20 mg/kg)	38,400 ± 800§	9,100 ± 400§
BCNU (20 mg/kg)/ Thymidine (2 g/kg)	39,800 ± 400§	31,300 ± 600¶

* Splenocytes from 3 mice were collected and pooled for each assay 12 days after drug treatment.

† Drugs were administered by a single i.p. injection.

‡ Each value refers to the [^{3}H]thymidine counts (mean ± SEM, N = 3).

§ $P < 0.0001$ vs control.

¶ $P < 0.4$ vs control.

TABLE 3. Antitumor cytotoxic activity of spleen cells from tumor-bearing mice*

Drug†	Days following drug treatment	Effector:Target cell ratio	% Specific [^{51}Cr]release‡ on: B16/F10	YAC-1	EL-4
Control (PBS)	12	50:1	0	0	0
		25:1	0	0	0
BCNU (20 mg/kg)	12	50:1	4	2	2
		25:1	1	0	1
BCNU (20 mg/kg)/ Thymidine (2 g/kg)	12	50:1	11	4	2
		25:1	8	2	1
BCNU (20 mg/kg)/ Thymidine (2 g/kg)	80	50:1	17	5	2
		25:1	9	2	1

* Spleen cells were obtained from either PBS-, BCNU-, or BCNU/thymidine-treated tumor-bearing mice 12 or 80 days after drug administration.

† Drugs were administered by a single i.p. injection, 24 hr after implantation of B16/F10 melanoma cells.

‡ Cytotoxicity was evaluated in a [^{51}Cr]release assay using B16/F10, YAC-1, or EL-4 as target cells. In each assay, triplicate samples were counted and averaged. Each assay was repeated two to three times.

BCNU-mediated suppression effects on immune-competent cells have been reported by other investigators [12]. In tumor-bearing mice, regeneration of immune-competent effector cells begins after approximately 1 week and is almost complete in 2 weeks following BCNU treatment [12]. During this period of depressed immunity, surviving tumor cells continue to proliferate. Our data indicated that the co-administration of BCNU and thymidine was less immunosuppressive, since splenocyte proliferation response to PHA was improved markedly over that of BCNU alone. It was also observed that following BCNU/thymidine treatment in normal mice, the percentage of CD8+ T-cells was increased (data not shown). Although the CD8 marker cannot distinguish suppressor T-cells from cytotoxic T-cells, the Con A-induced mitogenic response suggests that the suppressor T-cell population, rather than cytotoxic T-cells, was most likely selectively reduced by this treatment. This is consistent with the finding that in tumor-bearing mice the low tumor specific CTL activity after BCNU was increased significantly when thymidine was added.

Besides the effects on cytotoxic T-cell activity, helper/inducer activity (CD4+) may also be enhanced, as suggested by the enhanced DTH responses of BCNU/thymidine-cured mice. The activity of these cells may be enhanced as a result of reduced suppressor T-cell activity, which was suggested by the reduced Con A-induced mitogenic response [30]. Since no surviving mice after BCNU treatment were available for these DTH tests, it is unclear whether thymidine co-administration is required to generate the enhanced helper/inducer activity. Further studies of the status of suppressor T- as well as helper/inducer T-cell activity in the BCNU/thymidine-cured mice are required to clarify the mechanism underlying their enhanced DTH response.

Additional studies will include a determination of whether the immunity can be transferred, as well as the efficacy of BCNU/thymidine for metastatic tumors. Further proof of our hypothesis that the T-cell dependency for the BCNU/thymidine effect would be provided by a comparative evaluation of mice depleted of $CD4^+$ cells with mice depleted of CD8+ T cells. In a recent phase I study of sixty patients with refractory malignancies, who received various combinations of BCNU and thymidine, only one had a partial response [31]. In view of the unexpectedly low response rate and lack of myelosuppression and pulmonary toxicity at the highest dose of BCNU, a protective effect of thymidine was considered.

In summary, our findings have provided the first evidence for immune modulatory actions of thymidine by enhancing cytotoxic and helper/inducer T-cell activities as well as in reducing suppressor T-cell activity when used in combination with BCNU. The mechanism by which thymidine acts appears to reside in a preferential protection of all immune effector cells besides suppressor T-cells from the anti-proliferative and immunosuppressive action of BCNU. These immunomodulatory effects of thymidine suggest the potential usefulness of this drug, together with nitrosourea, in the therapy of patients with melanoma, and, possibly, other solid tumors.

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