Combined cyclotron fast-neutron and BCNU therapy in a rat brain-tumor model

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The combination of cyclotron fast-neutron radiotherapy with BCNU chemotherapy was compared to $^{137}$Cs gamma photon radiotherapy combined with BCNU in the 36B-10, F-344 rat-transplanted glioma model. Radiation and drug treatments were administered 7 to 8 days after intracerebral tumor implantation. Increase in animal survival time was used as the measure of the effectiveness of various treatment schedules.

Single-dose neutron or gamma radiotherapy was tested on Day 7 over the ranges 0 to 900 rads and 0 to 2000 rads, respectively. This therapy produced increases in mean survival times up to 70% at the highest radiation doses. When BCNU (10 mg/kg body weight) was administered intravenously on Day 8, 1 day following radiotherapy, mean survival times were increased by an additional 35% to 50%, irrespective of the dose or type of irradiation. In contrast, by using the same radiation and drug doses but scheduling combined therapy trials so that BCNU was administered 1 hour before either neutron or gamma irradiation on Day 7, there was enhancement of the radiation effect by BCNU. Under these conditions, the maximum enhancement of the mean survival time was 70% to 75% in neutron-treated animals and 120% to 150% in gamma-treated animals. Treatment with BCNU 1 hour before or 1 day after neutron irradiation proved to be no more effective in improving the survival time of tumor-bearing animals than the drug similarly combined with conventional gamma irradiation.

KEY WORDS - experimental tumor model - glioma - neutron radiotherapy - BCNU

THE standard therapy of supratentorial malignant astrocytic gliomas presently consists of surgical excision, which is usually subtotal, followed by a course of high-dose (5500 to 6000 rads), fractionated, whole-brain photon irradiation. This method of treatment yields a median survival time of 35 weeks. Brain-tumor therapists are now evaluating a considerable number of chemotherapy protocols aimed at improving the results of this therapy. From this work, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has emerged as the leading single drug in the treatment of malignant astrocytic gliomas because its administration has produced a response rate of 51% with a duration of 9 months in phase II trials, and because its use as an adjuvant to surgery and radiotherapy in phase III trials appears to lengthen median survival up to 50 weeks.

Concurrently, innovations in radiotherapy of malignant astrocytic gliomas are also being explored. Of particular interest is treatment with cyclotron-generated fast neutrons. The rationale for this mode of therapy is based 1) on the supposition that malignant astrocytic gliomas contain a substantial fraction of hypoxic tumor cells adjacent to regions of spontaneous tumor necrosis, and 2) on the fact that fast-neutron irradiation has a greater capacity than conventional photon beams to produce lethal damage in hypoxic tumor cells. Laramore, et al., recently reported their findings in 37 patients treated with fast-neutron or mixed neutron/photon irradiation. Neutrons proved to be no more effective in improving the length or quality of survival than conventional gamma irradiation. Nevertheless, postmortem examinations in 14 of 15 of these cases demonstrated extensive coagulative necrosis of the brain tumors. Scarcely any malignant-appearing tissue remained. Although there was evidence of radiation injury in tumor-free brain tissue, these pathological findings suggested that fast neutrons are highly tumoricidal in...
malignant astrocytic glioma therapy. Therefore, we have extended the assessment of neutron radiotherapy in a rat brain-tumor model, and now add a report on the preclinical evaluation of therapy with fast neutrons combined with the leading anti-glioma drug, BCNU.

Materials and Methods

Brain-Tumor Model

The rat brain-tumor model used in this work has been reported in detail elsewhere. Briefly, 5 × 10⁴ viable cells from in vitro stocks of an ethylnitrosourea-induced F-344 rat astrocytic glioma, numbered 36B-10, are transplanted into the right cerebral hemisphere of syngeneic females weighing 140 to 160 gm.* The exact stereotaxic injection coordinates are: 6 mm deep, 3 mm to the right of the midline, and 4.5 mm anterior to the frontal zero plane. Following transplantation, the tumor-bearing rats are randomly divided into groups according to treatment and dose. Untreated animals generally survive 17 to 21 days and then succumb to the mass effects of intracranial focal tumor expansion.

Whole-Head Irradiation

For irradiation with either neutrons or cesium-137 (¹³⁷Cs) gamma photons, unanesthetized rats were restrained in tapered plastic holders that prevented head turning and movement out of the radiation field. All of the body caudal to the head was shielded. All control rats were sham-irradiated.

A ¹³⁷Cs source was used for gamma irradiation. The dose rate was 60 rads/min, with a source-to-midbrain distance of 35 cm. Neutrons of approximately 8 MeV mean neutron energy were produced at the University of Washington cyclotron by deuteron bombardment of a beryllium target. The neutron-treated animals were irradiated at a distance of 106 cm from the beryllium target at a dose rate of approximately 60 rads/min of neutron plus gamma irradiation.

Dosimetry

The ¹³⁷Cs γ-ray doses were measured with thermoluminescent dosimeters placed at the intracerebral site of the tumor of sacrificed animals. Neutron dosimetry was carried out with a 1-ml tissue equivalent (Shonka A-150-TE-plastic) ionization chamber placed at the potential animal position. Under these conditions, the deuteron target current was calibrated as a function of the ion-chamber dose. For actual animal irradiation, the calibrated deuteron target current was used to measure the neutron dose delivered. The reported neutron rad dose includes the 5% gamma contamination of the beam.

BCNU Chemotherapy

The BCNU was obtained from the Drug Development Branch of the National Cancer Institute, National Institutes of Health, and was stored at −70°C until used. It was dissolved in a carrier solution of 10% ethanol and 90% normal saline immediately before injection in the femoral vein of animals lightly anesthetized with ether. The animals that received no chemotherapy were injected with a drug-free carrier solution.

Animal Survival Analysis

All animals were examined daily. The day of death was recorded, and the length of survival was measured from the time of transplantation. Percent increase in survival was calculated using the following formula: where A = mean survival time of treated rats and B = mean survival time of control rats.

Results

Preliminary Experimental Results

Before we examined the effects of combining BCNU with either neutron or gamma irradiation, we performed several preliminary experiments to determine 1) the optimum time interval following transplantation at which to administer radiation and BCNU treatments and 2) the optimum BCNU dose level to combine with radiotherapy.

Whole-head exposures of 1800 rads of ¹³⁷Cs gamma rays were delivered to groups of animals at several time intervals after tumor implantation. This dose of radiation was selected because it does not cause animal death from acute oral mucosal radiation injury or early radiation-induced dental disease, and yet is high enough to prolong survival of 36B-10 glioma-bearing rats significantly, so long as it is administered before the animals reach a state of preterminal debilitation from tumor mass effects. In studies to rule out effects from oral mucosal injury and dental disease, we have observed that sham-transplanted rats treated with 1800 rads reduce food and water consumption by 50% to 75% for 10 days following irradiation, but rapidly resume normal consumption and live 100 or more days. Rats implanted with tumors

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*Rats obtained from Simonsen Laboratories, Gilroy, California, or Charles River Laboratories, Wilmington, Massachusetts.

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and treated 7 to 8 days later with 1800 rads show subsequently a similar pattern of decreased food and water intake which also returns to nearly normal levels before the rats go on to die from tumor growth.

Figure 1a shows the effect of increasing the time interval between transplantation and the administration of 1800 rads of gamma rays. Survival in groups of tumor-bearing animals steadily declined as the post-transplantation interval increased. At 7 to 8 days posttransplantation, this dosage increased survival approximately 75% compared to the sham-irradiated control animals.

Treatment of tumor-bearing rats with neutrons was similarly assessed at a dose level of 700 rads. The decision to use this dose was based on previous data that have demonstrated an RBE (relative biological effect) of approximately 3 for neutron treatment at 1 or 2 weeks in this brain-tumor model. Survival of tumor-bearing animals again steadily declined as the post-transplantation interval increased (Fig. 1b). As with 1800 rads of gamma irradiation, 700 rads of neutrons increased survival approximately 70% at 7 to 8 days following transplantation. At the tested doses of gamma rays and neutrons, these results indicate that the time-dependent effects of both types of radiation on survival manifest a nearly identical parallel downward decline with increasing time after implantation of the tumor.

Several groups of tumor-bearing animals were then treated with intravenous injections of BCNU (10 mg/kg body weight, 0.75 × LD10 in rats) at various time intervals between 1 and 17 days after tumor implantation. The increase in mean survival time was greatest in degree, between 40% and 50%, in the groups of rats that received BCNU at time intervals between 7 and 14 days (Fig. 1c). Drug therapy was less effective when it was administered earlier than 4 days or later than 14 days after transplantation. This type of time-dependent response to BCNU has been previously demonstrated in the mouse glioma 26 brain-tumor model.

Finally, the relationship of the response to BCNU therapy as a function of dosage was determined by administering the drug in single doses from 5 to 50 mg/kg to rats bearing 8-day-old tumor grafts. Figure 2 shows that mean survival times increased with increasing BCNU dosages up to 20 mg/kg body weight, and then leveled off with no significant further increase in survival times at higher drug doses.

**Results of Later Experiments**

These preliminary results served as guidelines for further experiments designed to compare combined gamma irradiation plus BCNU to neutrons plus BCNU. Specifically, we elected to administer treat-
FIG. 2. Dose-response curve for the percentage increase in mean survival times of groups of intracerebral tumor-bearing rats treated with BCNU 8 days after tumor implantation. The numbers in parentheses represent the number of animals per group treated with the indicated BCNU doses. Bar symbols represent standard errors. The mean survival time in the sham-treated group was 19.0 ± 1.1 days.

FIG. 3. Dose-response curves for the percentage increase in mean survival times of intracerebral tumor-bearing rats treated with single doses of 137Cs gamma rays alone or in combination with BCNU (10 mg/kg body weight). Curve a, circles: BCNU administered 1 hour before irradiation on Day 7 posttransplantation. Curve b, squares: Radiation on Day 7 followed by BCNU on Day 8. Curve c, triangles: Radiation alone on Day 7. Each treatment group contained six to eight animals. Survival in the sham-treated control group was 18.5 ± 0.9 days. Bar symbols represent standard errors.

A dose of BCNU of 10 mg/kg body weight was selected for two principal reasons. This dose approximates the maximum antitumor effect attainable with BCNU in our brain-tumor model; and the likelihood of producing toxicity by combining BCNU with high radiation doses is less with 10 mg/kg than it is at higher drug doses.

BCNU Combined with 137Cs Gamma Irradiation. Groups of tumor-bearing animals treated on Day 7 with gamma radiation in the dose range 0 to 2000 rads, and on Day 8 with BCNU, demonstrated increases in mean survival times that were greater than the increases recorded in animals that received radiation alone on Day 7 (Fig. 3, curves b and c). At all radiation dose levels, except 2000 rads, the increase in mean survival time with added BCNU treatment was 35% to 50% greater than in animals that were only irradiated (p < 0.01). Animals that received BCNU but no radiotherapy also showed a 50% increase in survival time. This experiment was repeated twice with similar results.

On the other hand, when BCNU treatment was administered 1 hour before gamma irradiation delivered over the same dose range as above (Fig. 3, curve a), the increases in animal survival times were 100% to 150% greater between 900 and 1800 rads than produced by gamma irradiation alone (Fig. 3, curve c: p < 0.01). Over the same radiation dose range, these increases (curve a) were also greater than we observed when BCNU was given 1 day after irradiation (curve b: p < 0.01). Thus, administering BCNU before gamma radiotherapy enhanced the effect of irradiation on...
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The maximum enhancement of irradiation recorded when BCNU was administered 1 hour before gamma rays was 120% to 150% at the 1400- and 1800-rad doses. In contrast, the maximum enhancement observed when BCNU was administered 1 hour before neutrons was 70% to 75% at the 700- and 900-rad doses. Comparing these maximum effects at these doses reveals that the BCNU-gamma irradiation combination augmented survival greater than the BCNU-neutron combination ($p < 0.05$).

**Discussion**

The survival data on groups of intracerebral transplant-bearing rats treated on Day 8 with single doses of BCNU over the range of 0 to 50 mg/kg indicate that the tumoricidal effect of BCNU levels off appreciably above the 20 mg/kg dose level (Fig. 2). Rosenblum and co-workers$^{13,18}$ noted a similar dose-response effect of BCNU in the 9L F-344 rat gliosarcoma model. These workers assayed *in vitro* the survival of clonogenic 9L cells harvested from intracerebral implants treated *in vivo* with single doses of BCNU over the range of 0 to 27 mg/kg. They observed that a dose of 10 mg/kg reduced the surviving fraction of clonogenic tumor cells to approximately $10^{-8}$, a three-log-cell kill, whereas at higher doses the surviving fraction was not further reduced significantly.

Although these observations in the two brain-tumor models confirm the potent tumoricidal effect of single-dose BCNU therapy, it remains unclear why a small, yet eventually lethal, fraction of tumor cells survive despite dose increments above a certain level, that is, the 10 to 20 mg/kg range. There may be cell cycle kinetic, biochemical, or drug delivery effects that individually or in concert operate to protect a select population of cells. For the following reasons we agree with Rosenblum, et al.$^{17}$ that the effectiveness of BCNU is in part limited by drug delivery problems. Histological studies by us and others$^{8}$ have revealed that nascent tumors lack intrinsic capillary channels during the first few days following glioma cell implantation in brain. Coincident with this lack of a vascular network in the tumors, the effectiveness of BCNU is demonstrably lower (Fig. 2) than it is after the 7-day posttransplantation time point when intrinsic tumor vasculature is developed. These findings suggest that nascent tumors lack a vascular network sufficient to permit tumoricidal levels of BCNU to reach the entire population of tumor cells. With further development of the implants, it is likely that drug delivery improves as vascular proliferation ensues, but not to such a degree that the drug can reach all the tumor cells in cytotoxic amounts. This postulation cannot be nullified by arguing that nascent intracerebral glioma implants consist chiefly of nonproliferating cells$^{4}$ which are resistant to otherwise tumoricidal levels of BCNU. That BCNU has the capacity to produce a three-log-cell kill in 9L gliosarcoma during exponen-
tial growth *in vivo* when 50% of the tumor cells are in the nonproliferating pool clearly establishes that nonproliferating cells are killed by this drug when they are exposed to it.\(^{17,18}\)

In contrast to BCNU, single-dose \(^{197}\)Cs gamma or neutron irradiation increased animal survival to the greatest degree when it was administered during the nascent period of tumor growth (Fig. 1a and b). Delivery of these modes of therapy is not dependent on a functioning tumor vascular network. However, the effectiveness of either single-dose BCNU or irradiation rapidly waned during the preterminal phase of intracerebral tumor growth (Fig. 1), that is, 14 days and later after transplantation. This is explained by slow dead cell removal,\(^{11}\) continued proliferation of clonogenic cells, and the presence of intratumoral and intracerebral edema, all of which contribute to an increasing mass effect, cerebellar tonsillar herniation, and rapid death.

The chief purpose of this investigation was to explore whether the combination of fast neutrons plus BCNU is more effective treatment of intracerebral 36B-10 glioma grafts than gamma photons plus BCNU. Our findings have established that combining BCNU at 10 mg/kg body weight with single-dose neutron or gamma radiotherapy produces a substantially longer duration of animal survival than is produced by the administration of either type of radiation alone. This extension of animal survival due to BCNU occurred at all radiation doses; it was observed when the drug was administered 1 hour before or 1 day after irradiation. However, BCNU combined with fast-neutron therapy did not demonstrate greater augmentation of animal survival than it did when combined with gamma irradiation.

The scheduling sequence of drug with respect to irradiation had an important effect on the extent to which animal survival was increased. Administering single-dose BCNU 1 hour before either gamma or neutron irradiation proved to be decidedly more effective than administering the drug 1 day after irradiation. Mealey, *et al.*,\(^{10}\) reported that BCNU treatment of human glioblastoma multiforme cells *in vitro* enhanced the sensitivity of these cells to subsequent treatment with single graduated doses of x-irradiation. These findings have been extended by Wheeler, *et al.*,\(^{26,27}\) and Deen, *et al.*,\(^{6}\) who examined the combined effects of BCNU and x-irradiation on rat 9L gliosarcoma both *in vitro* and *in vivo*. Treatment of 9L cells *in vitro* with BCNU at several time intervals from 1 to 23 hours before treatment with graduated single doses of x-irradiation enhanced x-ray cell kill significantly, especially when the time interval between BCNU and irradiation treatment was 15 to 16 hours. Treatment with BCNU after irradiation did not produce enhancement of the radiation effect. A similar time-dependent interaction of this drug and x-irradiation was defined *in vivo* when fractionated BCNU treatments of 2 mg/kg body weight were administered at either 2-, 6-, or 16-hour intervals before daily x-irradiation fractions of 260 rads.\(^{27}\) Our results with both single-dose gamma irradiation (Fig. 3, curve a) or neutron irradiation (Fig. 4, curve a) administered 1 hour after BCNU treatment are thus consistent with the findings of others who have shown that BCNU enhances the effect of irradiation when it is administered prior to the irradiation at certain time intervals. To produce this enhancement effect, BCNU may efface repair of sublethal or potentially lethal radiation damage, or alternatively it may arrest proliferating cells at radiosensitive periods of the cell cycle.\(^{6,9,26}\)

On the other hand, Barker, *et al.*,\(^3\) did not find that survival of 9L gliosarcoma-bearing rats was greater when BCNU was administered before irradiation compared to drug treatment after irradiation. However, in these experiments treatments were applied at a relatively later time in the course of tumor growth than we and others\(^{27}\) examined. As Barker, *et al.*,\(^3\) and Wheeler, *et al.*,\(^{27}\) suggest, these disparate results may be related to differences in the tumor growth kinetics extant when treatment is begun.

It does not appear from our results at 7 to 8 days posttransplantation that BCNU given 1 hour before neutrons leads to any greater enhancement of the radiotherapy response than BCNU given before gamma irradiation. In fact, the findings shown in Figs. 3 and 4 suggest rather that BCNU administered prior to gamma photon irradiation produces greater enhancement of animal survival than BCNU given 1 hour before neutron irradiation. Our observations suggest that combining BCNU therapy with conventional gamma irradiation may be equally or possibly more effective than BCNU combined with fast neutrons in the treatment of malignant astrocytic gliomas. It will be necessary, however, to do further studies, particularly of multiple fractions of drug and radiation, to verify this point.

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**References**

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