Journal of Neuro-Oncology **46:** 231–240, 2000.

Laboratory Investigation

The combination of boron neutron-capture therapy and immunoprophylaxis for advanced intracerebral gliosarcomas in rats

H.M. Smilowitz[1], P.L. Micca[2], M.M. Nawrocky[2], D.N. Slatkin[1,2], W. Tu[1] and J.A. Coderre[2]
[1]*Department of Pharmacology, University of Connecticut Health Center, Farmington, CT, USA;* [2]*Medical Department, Brookhaven National Laboratory, Upton, NY, USA*

Key words: BNCT, glioma, immunoprophylaxis, immunological memory, radiotherapy

Summary

Glioblastoma multiforme (GBM) is the most common primary human brain tumor. About 7000 new cases are diagnosed yearly in the USA and GBM is almost invariably fatal within a few years after it is diagnosed. Despite current neurosurgical and radiotherapeutic tumor cytoreduction methods, in most cases occult foci of tumor cells infiltrate surrounding brain tissues and cause recurrent disease. Therefore the combination of neurosurgical and radiotherapeutic debulking methods with therapies to inhibit occult GBM cells should improve prognosis. In this study we have combined boron neutron-capture therapy (BNCT), a novel binary radiotherapeutic treatment modality that selectively irradiates tumor tissue and largely spares normal brain tissue, with immunoprophylaxis, a form of active immunization initiated soon after BNCT treatment, to treat advanced, clinically relevantly-sized brain tumors in rats. Using a malignant rat glioma model of high immunogenicity, the 9L gliosarcoma, we have shown that about half of the rats that would have died after receiving BNCT debulking alone, survived after receiving BNCT plus immunoprophylaxis. Further, most of the surviving rats display immunological-based resistance to recurrent 9LGS growth six months or more after treatment. To our knowledge this study represents the first time BNCT and immunoprophylaxis have been combined to treat advanced brain tumors in rats.

Abbreviations: GBM – glioblastoma multiforme; BNCT – boron neutron-capture therapy; 9LGS – 9L gliosarcoma; FDA – Food and Drug Administration; BNL – Brookhaven National Laboratory; UCHC – University of Connecticut Health Center; CSF – cerebro spinal fluid; SD – standard deviation; i.c. – intracerebral; s.c. – subcutaneous; BMRR – Brookhaven Medical Research Reactor.

Introduction

Glioblastoma multiforme (GBM), the most common of the malignant astrocytomas in adults, is newly diagnosed in about 7000 Americans (median age 59) every year. At the time of diagnosis, GBM is usually over 3 cm in diameter. Surgical removal of the visible tumor mass with non-eloquent portions of the adjacent edematous brain microscopically infiltrated with tumor cells, when possible, alleviates the imminent risk of death from brain swelling. Postoperative photon- or proton-based radiotherapy provides further life extension. But GBM, which in adults is usually a unilateral, unifocal neoplasm of the cerebrum, usually recurs within 3 cm of its original margin from microscopic nests of cells that infiltrate the surrounding edematous brain tissue [1]. Median survival is only about ten months using standard photon-based radiation therapy; five year survival is less than 5%. Aggressive photon-based radiotherapy needed to kill clonogenic cells several centimeters beyond the macroscopic periphery of the tumor and thereby prolong survival, however, increases the probability of severe neurological side-effects. For these reasons, new approaches to GBM therapy are needed. One such approach, boron neutron-capture therapy (BNCT), is an experimental radiation therapy.

BNCT [2–5, for reviews] is a binary treatment modality that can selectively irradiate tumor tissue. BNCT uses drugs containing a stable isotope of boron,

^{10}B, to sensitize tumor cells to irradiation by low energy (thermal) neutrons [6–10]. The interaction of the ^{10}B with thermal neutrons (neutron-capture) causes the ^{10}B nucleus to split, releasing an alpha particle and a lithium nucleus. These products of the ^{10}B(n,α)^{7}Li reaction, which are very damaging to cells [11–13], have a combined path length in tissue of approximately 14 μm [14], roughly twice the diameter of a dividing GBM cell. Thus, most of the ionizing energy imparted to tissue is localized to ^{10}B-loaded cells so that it is the biodistribution pattern of the boron compound that is the key to the effectiveness of BNCT. An FDA-approved clinical trial of BNCT for patients with GBM was initiated at BNL in September 1994; this trial, now in a dose escalation phase [15,16] has achieved clinically useful palliation of most of the 39 reported cerebral GBM patients treated by *p*-boronophenylalanine-based BNCT after neurosurgical debulking of the tumor [16]. BNCT may offer some advantages for post-surgical debulking over conventional radiotherapy and chemotherapy [4,5,17]. Although the median time to recurrence of the tumor (12–15 months) has been improved only slightly over that which is achieved from conventional post-debulking radiotherapy [9–10 months], BNCT is administered in one fraction; standard post-operative radiotherapy for GBM requires thirty fractions over six weeks necessitating weeks of travel to a radiooncology center. Furthermore, BNCT can provide some patients with a superior quality of remaining life due to exceptional sparing of normal CNS tissue. Like standard post-operative radiotherapy, BNCT spares the patient's immune system. However, although the radiation risk (sieverts) of BNCT implemented at a nuclear reactor is comparable to that of conventional gamma therapy [18], the scattered dose to the body is greater from gamma rays than from the mixed radiations from the reactor treatment directed to the head. Therefore the systemic radiation to the body's lymphopoietic system may be less in total dose and less prolonged in time from BNCT than from conventional post-operative radiotherapy. BNCT is currently undergoing further development and optimization, primarily for brain tumors [9,19]. Although in theory BNCT can target all tumor cells, it is likely that in human GBM, zones of poorly vascularized tumor may receive suboptimal amounts of boronated drug. Statistically, it is likely that some tumor cells may not be hit by any particle released during boron neutron-capture reactions [20]. Therefore post-debulking BNCT combined with other forms of GBM therapy to destroy remaining cells should prove more efficacious for human GBM than BNCT alone. In this paper we have modeled one such combination by comparing BNCT and immunoprophylaxis (i.e. prophylactic immunotherapy) with BNCT alone to treat intracerebrally transplanted, advanced gliosarcomas in rats.

There have been several reports of active immunotherapy of human gliomas with and without standard radiotherapy [for review, 21] which were well tolerated, resulted in positive delayed hypersenstivity reactions [22–26] and, in some studies, provided minor extensions of life. Relatively recent advances in the cell and molecular biology of immunity have spurred renewed interest in immunotherapy of brain tumors [27]. There has also developed an interest in using genetically modified tumor cells as immunogens [28]. However it appears to us that experimental brain tumor immunotherapy is often initiated when the animal's brain tumor is too small relative to the animal brain under study to infer clinical relevance due to the disproportion of scale with that appropriate for human tumors at the time of clinical treatment relative to the size of the human brain. In this paper we combine BNCT and prompt, post-BNCT immunoprophylaxis to treat large, imminently lethal (about 1–2 weeks prior to death), clinically relevantly-sized experimental brain tumors (i.e. about 40 $\pm$ 20 mg) of high immunogenicity, which occupy several percent of the volume of the test rat's cranium. This investigation should provide a frame of reference for subsequent studies of immunoprophylaxis in rat glioma models of lower immunogenicity, which we believe will lead to analogous clinical immunoprophylaxis to help avert recurrence of human malignant astrocytomas.

Materials and methods

Cell lines and animals

The transplantable rat 9L gliosarcoma (9LGS) cell line was originally induced in a Fischer 344 rat by weekly intravenous (i.v.) injections of N-nitrosomethyurea [29] and susequently serially passaged *in vitro* at the Department of Medicine, Montefiore Medical Center, Bronx, NY by Dr. Victor Hatcher before transfer in the mid-1980s to the late Dr. Ralph G. Fairchild at the Medical Department (BNL). The 9LGS/BNL strain was transferred to H.M. Smilowitz (UCHC) by J.A. Coderre (BNL) in 1996. Pathogen-free male

Fischer 344 rats (~ 8–12 weeks old) (Taconic Farms, Germantown, NY) were used as indicated.

Anesthesia

For BNCT/immunoprophylaxis experiments, rats were anesthetized for tumor implants or surgical procedures with an intraperitoneal (i.p.) injection of 54 mg/kg ketamine and 9 mg/kg xylazine. For the graded challenge response curves, rats were anesthetized by isofluorane anesthesia using a Narkomed 2 anesthesia machine (North American Drager, Telford, PA) for the subcutaneous (s.c.) implantation of tumor cells.

Cell culture

Cells were maintained at UCHC in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum (Hyclone) and Pen-Strep (GIBCO), removed from tissue culture flasks with trypsin/EDTA solution (GIBCO) and used between passages 12 and 25.

Subcutaneous implantation of 9LGS cells

For the initiation of s.c. tumors, 9LGS cells were removed from T75 flasks (Sarstedt). Cell clumps were mechanically disrupted using a Pasteur pipet; 5,000,000 cells were suspended in 0.1 ml of medium and injected s.c. in the left flank of an anesthetized rat through a 25 gauge needle. Over the course of each experiment, > 95% of such cells proved viable by a trypan blue dye-exclusion test.

X-irradiation of 9LGS cells

A suspension of 100,000,000 9LGS cells in 2 ml was placed into a 5 ml glass injection vial, then irradiated through a 2.5-cm diameter collimator aperture for 10 min with Cu/Al-filtered 100 kVp X rays (6.60 Gy/min). The physical absorbed dose delivered to the cells thereby was 50 Gy. Alternatively, for the graded challenge response curves, cells were irradiated (0.765 Gy/min) in a cesium 137 Gamma Cell 40 Irradiator, Atomic Energy of Canada (Nordion).

Surgical removal of subcutaneous 9LGS tumors

Subcutaneous 9LGS tumors were excised 11–12 days after implantation from anesthetized rats using asceptic surgical techniques. Rats were anesthetized with ketamine/xylazine and the surgical field was shaved and disinfected. By day 11, tumors weighed an average of 865 mg and appeared larger than ~ 1 cm^2 in surface area tangential to the skin. Approximately 75% of the tumors were mobile and operable; ~ 15% of the tumors were fixed and too deep to remove; those rats were euthanized. The other ~ 10% had begun to invade the underlying muscle but were not too deep to be excised.

Intracranial implantation of 9LGS cells

Tumors were initiated in anesthetized rats weighing about 200 g [6,7,12] by inoculating 1 μl of culture medium containing 10,000 cultured 9LGS cells (> 95% viable) into the left striatum 4–5 mm deep at a point 4 mm to the left of the midline along the (serated) coronal suture. A 0.5-mm burr hole was drilled at that point through the skull. A 27-gauge needle, which was fitted with a depth-limiting plastic collar to ensure cell injection 4–5 mm beneath the skull, was connected to a Hamilton microsyringe (Las Vegas, NV) via flexible tubing. Following a 30-sec infusion of the cells, another 30 sec was allowed for the cells to settle before removing the inoculation needle from the brain. This technique resulted in a locally expanding tumor of the striatum and around the inoculation needle track with no evidence of blood- or CSF-borne metastases although, like human glioblastoma [30], this tumor can seed the ventricles if inoculated more caudally in the striatum. Death ensues 21 ± 3 (mean ± SD) days after inoculation from intracerebral tumor growth [7]. For intracerebral (i.c.) contralateral rechallenge, 10,000 cultured 9LGS cells in 1.0 μl of culture medium were injected similarly 4 mm to the right of the midline along the coronal suture.

Boron neutron-capture therapy

For rat brain tumor irradiations, the beam emerging from the Brookhaven Medical Research Reactor (BMRR) thermal neutron port was restricted to a 2.0 cm-diameter aperture by a 10.16 cm-thick collimator made of $^{6}LiCO_3$ dispersed in polyethylene (Li-poly), molded with a centered conical aperture of 12 cm diameter tapering to 2 cm diameter [31]. BNCT of rats bearing intracerebral 9LGS was carried out 14 days after implantation when the tumors were approximately 4 mm in diameter [7,12]. Rats were anesthetized for the BMRR irradiations with an i.p. injection of

ketamine (54 mg/kg) and xylazine (9 mg/kg). Each anesthetized rat was placed supine and perpendicular to the thermal neutron port with the tumor zone centered in the 2 cm aperture. A blood sample was obtained from each rat at the time of irradiation for boron analysis and dosimetric calculations. After the irradiation, rats were observed daily and body weights were recorded at least three times per week for the first few weeks and at least weekly thereafter. Groups of rats irradiated on different days were combined for analysis. A control group of unirradiated, concurrently implanted 9LGS-bearing rats was included with each series of rats irradiated on one day.

Measurements and statistics

Subcutaneous tumor volumes in mm^3 were calculated using orthogonal tumor dimensions in mm (width [W] and length [L]) according to $W^2L/2$ [32].

A measure of the likelihood that there is a statistically significant difference between groups of animals, the confidence level p, was evaluated by the non-parametric Wilcoxon Two-Sample test with reference to published tables of acceptance regions for the ties-corrected rank sum for p values in the range of 0.1–0.001 [33]. P^* represents the confidence level for the comparison with a group comprising the control group in the indicated experiment and the control group in a similar experiment.

Proportions of surviving rats were compared using a χ^2test [34].

Results

The combination of immunoprophylaxis and BNCT for advanced rat brain tumors

After surgical reflection of the scalp, 10,000 9LGS cells were injected intracerebrally in the left striatum in 1 μl of culture medium [6,7,12] as described in Methods. Fourteen days later, when these untreated brain tumors were expected to be about 4 mm in diameter (∼ 40 mg) [6,7,12], the rats were anesthetized and the tumors were irradiated at the thermal neutron port of the BMRR for 3.15 MW-min (day 0). The animals were injected with a total dose of 1200 mg BPA/kg body weight prior to BNCT [13]. These conditions provide sub-optimal BNCT in which approximately 1/2 of the rats are expected to survive long-term (i.e. > 6 months). After irradiation on day 0, one group of rats received no further treatment (BNCT only, group 2); a second group of rats was not treated by BNCT (untreated control, group 1); a third group received BNCT on day 0 followed by a single s.c. injection of 5,000,000 cultured 9LGS cells into their left thigh on day 0 and the resulting tumors were excised surgically on day 11 (group 3); the fourth group of rats received BNCT on day 0 followed by a series of s.c. injections of 5,000,000 cultured and then irradiated (50 Gy) cells into their left thigh on days 0, 7, 21, 35, 49, 63 (group 4). A fifth group of rats were not treated by BNCT and were similarly injected s.c. with 5,000,000 cultured and irradiated cells into their left thigh (group 5) on day 0. Table 1, the combined results of four separate trials, shows that none of the rats survived long-term in the two groups not treated by BNCT (group 1, median survival = 6 days

Table 1. The combination of immunoprophylaxis and boron neutron-capture therapy for advanced rat brain tumors

Group	*N*	Surviving fraction	Percent (%)	Death (days following BNCT/immuno-prophylaxis)	Median (days)
1	20	0/20	0	3, 4, 4, 4, 5, 6, 6, 6, 6, 6, 6, 6, 7, 7, 7, 8, 11, 11, 12, 18	6
2	43	26/43	60	18, 25, 25, 28, 32, 33, 33, 33, 34, 36, 39, 45, 48, 50, 51, 78, 95	34
3	27	21/27*	78	25, 26, 32, 47, 49, 54	39.5
4	33	28/33**	85	18, 22, 25, 28, 35	25
5	14	0/14	0	4, 4, 4, 5, 5, 5, 5, 5, 6, 6, 6, 8, 10, 10	5

Group 1: untreated; group 2: BNCT only; group 3: BNCT + surgery of s.c. viable tumors; group 4: BNCT + multiple injections of irradiated cells; group 5: multiple injections of irradiated cells.

Fischer 344 rats received a 1 ml intracerebral (i.c.) injection of 10,000 9LGS cells 14 days before treatment (see Methods). Fourteen days later, day 0: group 1 no further therapy; group 2, sub-optimal BNCT alone (see Methods); group 3, sub-optimal BNCT plus a single injection of 5,000,000 live 9LGS cells. The resulting tumors were removed surgically eleven days later; group 4, sub-optimal BNCT plus multiple injections of 5,000,000 irradiated (50 Gy) 9LGS cells injected on days 0, 7, 21, 35, 49, 63. Group 5, multiple injections of 5,000,000 irradiated (50 Gy) 9LGS cells as above. The data of groups 1, 2, 3, 4 and 5 represent the combined results of 4, 4, 3, 3 and 1 similar trials implemented at different times, respectively. $^*\chi^2$ (1 degree of freedom) = 2.253, $p = 0.133$; $^{**}\chi^2$ (1 degree of freedom) = 5.397, $p = 0.02$.

after BNCT treatment of the other groups; group 5, median survival = 5 days following immunotherapy); immunotherapy without BNCT was ineffective. However, sixty-three percent of the rats treated by BNCT alone survived for six months, at which time they were used for additional experiments. The median survival time following BNCT and the percent long-term survival in the BNCT-only group were 34 days and 60%, respectively. Rats treated by the combination of BNCT and live-cell immunoprophylaxis (group 3) yielded 78% long-term survival. The median survival time of the rats that died was 39.5 days. χ^2test (one degree of freedom) = 2.253, $p = 0.133$. BNCT and multiple injections of irradiated cells (group 4) yielded 85% long-term survival; the median survival time of the rats that died was 26.5 days. χ^2 test (one degree of freedom) = 5.397, $p = 0.02$. In one of the four trials, a group of eight rats received only a single s.c. injection of 5,000,000 cultured and then irradiated (50 Gy) 9LGS cells into their left thigh on day 0. Of these rats, only three survived (37%), which is similar to the group that received BNCT only in that experiment, i.e. 4/8 (50%).

Table 2. Intracerebral rechallenge of six-months surviving rats with 9LGS

Group	*N*	Surviving fraction	Percent (%)	Death/euthanasia (days following rechallenge)
1	5	0/5	0	4, 5, 10, 10, 38
2	6	2/6	33	5, 5, 17, 34
2A	12	4/12	33	2, 3, 5, 5 8, 9, 17, 34
3	4	4/4	100	None
4	3	2/3	66	10
3 and 4	7	6/7	86	10

Group 1: untreated control; group 2: six-month survivors, BNCT-only; group 2A: six-month survivors, BNCT-only (combined with previous data (35)); group 3: six-month survivors, BNCT and viable cell therapy; group 4: six-month survivors, BNCT and single injection of X-irradiated cell therapy.

Six-month surviving rats from Table 1 were rechallenged with 10,000 9LGS cells i.c. on the contralateral side (Methods) and followed for survival. Group 1 rats were untreated six to eight week old rats which served as a control for the i.c. cell injections. Group 2 rats were BNCT-only survivors (Table 1 Group 2). Group 2A represents combined data from group 2 (above) and data from a similar group (35). Group 3 rats were six-month survivors which had received sub-optimal BNCT plus a s.c. injection of viable 9LGS cells forming s.c. tumors that were removed surgically. Group 4 rats were six-month survivors which had received sub-optimal BNCT therapy plus a single injection of x-irradiated 9LGS cells.

Rechallenge of surviving rats with intracerebral 9LGS cells

Some of the rats that had survived their implanted 9LGS tumors six months after therapy were rechallenged by i.c. injection of 9LGS cells symmetrically into the contralateral, i.e., the right side of the brain. Table 2 shows the survival of such rechallenged rats. Whereas none of the untreated control rats (group 1) survived 9LGS i.c. injections, two of the six rats that had received BNCT therapy alone for their advanced brain tumors (group 2) survived 9LGS rechallenge, as observed in a previous experiment [35]. The data from the previous and the present experiments have been combined (group 2A). All of the six-month surviving rats that received BNCT plus viable-cell immunoprophylaxis (followed by the surgical removal of the ensuing s.c. tumors) (group 3) and were rechallenged ($N = 4$) survived the i.c. rechallenge. Two of the three six-month surviving rats that had received a single injection of X-irradiated 9LGS cells (group 4) survived i.c. 9LGS rechallenge. The data from i.c. rechallenge of the six-month surviving rats that had received BNCT plus viable cell immunoprophylaxis (group 3) or X-irradiated cell immunoprophylaxis (group 4) show that those survivors exhibited better immunologic memory than did the six-month survivors that had received BNCT alone (group 2A) ($p < 0.01$).

Graded challenge response curve: immunoprophylaxis with unirradiated cells

Rats were injected with 5,000,000 untreated 9LGS cells s.c. in their left thigh. The resulting tumors (0.93 g ± 0.32 g; mean ± SD) were excised surgically eleven days later. One week thereafter, the rats were challenged by contralateral s.c. injections with increasing numbers of untreated 9LGS 'challenge' cells numbering 500,000–20,000,000. The ordinate of Figure 1 shows the proportion of rats in which the injected cells formed a progressively growing neoplasm (filled circles). Whereas s.c. challenge with 500,000 cells ($N =$ 6 rats) resulted in no progressively growing tumors, challenge with 5,000,000 ($N = 12$) and 10,000,000 ($N = 18$) cells resulted in tumors in approximately half of the rats; challenge with 20,000,000 cells ($N = 6$) resulted in tumors in 5/6 rats.

In an additional six rats, untreated 9LGS cells were first injected intradermally (i.d.) in the ipsilateral thigh.

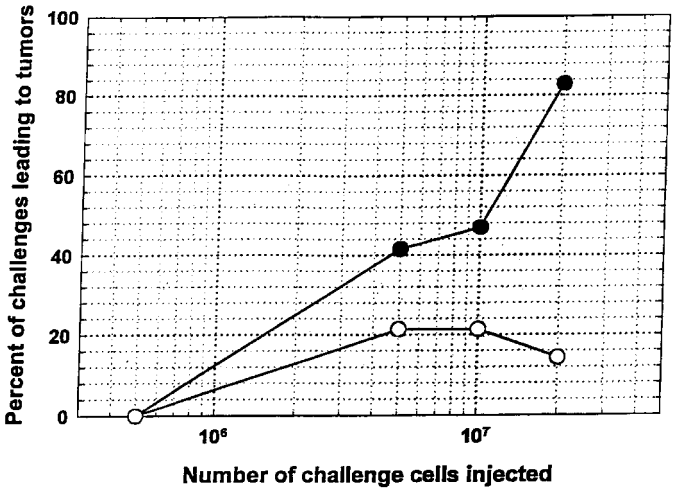

Figure 1. Graded challenge response curves. -●-: Immunoprophylaxis with unirradiated cells. Rats were injected with 5,000,000 untreated 9LGS cells s.c. in their left thigh (day-18). The resulting tumors were excised surgically eleven days later (day-7). One week thereafter (day 0) the rats were challenged with contralateral s.c. injections of increasing numbers (500,000–20,000,000) of parental 9LGS cells (abscissa). The ordinate shows the proportion of rats in which s.c. injected cells formed a progressively growing neoplasm. 500,000 cells, $N = 6$ rats; 5,000,000 cells, $N = 12$ rats (two independent trials); 10,000,000 cells, $N = 18$ rats (three independent trials); 20,000,000 cells, $N = 6$ rats. -○-: Immunoprophylaxis with multiple injections of irradiated cells. Rats were injected with 5,000,000 irradiated (50 Gy) 9LGS cells s.c. in their left thigh (day-18). One week later the rats were boosted with 5,000,000 irradiated (50 Gy) cells (day-11), a procedure that was repeated on days 21, 35, 49 and 63. On day 0 the rats were challenged with contralateral s.c. injections of increasing numbers (5,000,000–20,000,000) of parental 9LGS cells (abscissa). The ordinate shows the proportion of rats in which s.c. injected cells formed a progressively growing neoplasm. 5,000,000 cells, $N = 14$ rats (two independent trials); 10,000,000 cells, $N = 14$ rats (two independent trials); 20,000,000 cells, $N = 14$ rats (two independent trials).

Intradermal tumors (0.05 cc $\pm$ .04 cc mean $\pm$ SD), which were much smaller than s.c. tumors, were excised surgically eleven days later. These rats were then challenged on the contralateral side s.c. with 5,000,000 untreated 9LGS cells seven days thereafter. All of the resulting tumors grew progressively.

Graded challenge response curve: immunoprophylaxis with irradiated cells

Rats were injected with 5,000,000 cultured, irradiated 9LGS cells (50 Gy) s.c. in their left thigh. Tumors were seen and palpated within three days. Those tumors disappeared spontaneously over the next week. Seven days after the first injection, the rats were injected s.c. in the ipsilateral thigh again with 5,000,000 irradiated cells. Similar s.c. injections of irradiated 9LGS cells into the left thigh of these rats were performed on days 21, 35, 49 and 63. On day 18 after the first injection, the rats were injected s.c. (i.e., 'challenged') with various numbers of untreated 9LGS cells in the contralateral (i.e., right) thigh. Figure 1 (open circles) shows the proportion of rats in which the challenge cells formed a progressively growing neoplasm at the site of injection (ordinate) as a function of the number of cells injected (abscissa). On average, fewer than 1/4 of all rats challenged with 5,000,000 ($N = 14$), 10,000,000 ($N = 14$) and 20,000,000 ($N = 14$) untreated cells exhibited progressive tumor growth. These data suggest that immunoprophylaxis using multiple s.c. injections of irradiated 9LGS cells protects rats against a large s.c. tumor-cell challenge better than does a single injection of live 9LGS cells and better than does the surgical excision of the resulting tumor eleven days after viable cell immunoprophylaxis.

Discussion

We have combined BNCT and immunoprophylaxis to treat a clinically relevantly-sized experimental brain tumor of the rat. To our knowledge, this represents the first documentation of active immunoprophylaxis

to treat large, clinically relevantly-sized and imminently lethal brain tumors. Immunoprophylaxis is defined as active immunization to avert or delay tumor regrowth following prior tumor-debulking procedures such as surgical excision and/or local radiation therapy; 'clinically relevant' (i.e., imminently lethal) is defined here as a tumor so advanced that the residual life span of the concomitantly untreated control animals will be no more than 1/3 to 1/2 of the total time between tumor inoculation and death from tumor overgrowth in the brain. The untreated 9LGS typically causes death about three to four weeks after initiation under the conditions we used (see Methods); in the experiments reported here death was 21.2 ± 3.5 (mean $\pm$ SD) after cell implantation. Thus 'clinically relevant' experimental therapy was not begun until 14 days after tumor inoculation. Under these conditions, Table 1, group 6, clearly shows that immunotherapy alone, initiated on day 14 after tumor inoculation, provides no benefit over no treatment at all. There are numerous reports of experimental immunotherapy of brain tumors with more favorable outcomes in which therapy was initiated within one week of i.c. inoculation of tumor cells [36–57]. However we are not aware of other reports on immunotherapy or immunoprophylaxis of clinically relevant experimental gliomas in immunocompetent hosts. A recent report has demonstrated the efficacy of herpes virus therapy for genetically modified human GBM tumors in cyclophosphamide- and RMP7- treated athymic rats [58].

The 9LGS rat tumor is a well established rat brain tumor model which, although highly immunogenic, nevertheless bears some informative similarities to human GBM [7,29]. This model, as reported here, provides one with a frame of reference for the therapeutic benefit that can potentially be obtained for human brain tumors (e.g. GBM) when their demonstrably greater invasiveness and their putatively lower levels of tumor immunogenicity must be confronted. The level of immunogenicity of the 9LGS tumor model was quantified by the graded challenge response curves provided in Figure 1. Either immunization with live 9LGS cells followed by the excision of the ensuing tumors or the repeated immunization with X-irradiated tumor cells, which form pseudotumors that regress spontaneously following an initial week of growth [35], was shown to result in the rejection of contralateral s.c. challenge with live 9LGS cells. The immunogenicity index, defined as the concentration of challenge cells resulting in 50% rejection of the challenge, is shown to be 5,000,000–10,000,000 cells for unmodified 9LGS (Figure 1, closed circles (i.e. live cell immunization)) and $> 20,000,000$ cells (Figure 1, open circles (i.e. multiple injections of irradiated cells)) without immune enhancers such as GMCSF [59] and IL12 [60]. With this immunogenicity index, we have shown that if the BNCT dose is such that 60% of the rats survive their brain tumors for > 1 year (40 Gy-Eq), the injection of live 9LGS cells s.c. at the time of BNCT followed by the surgical excision of resulting tumors increases survival to 78%. Similarly, multiple injections of X-irradiated 9LGS cells s.c. at the time of BNCT, so that tumors grow and then spontaneously regress, increases survival to 85%. More than half of the rats that would have died had they received no treatment other than BNCT have survived for a year. The χ^2 test (one degree of freedom) shows that the increase in survival attributable to the combination of immunoprophylaxis (multiple injection of irradiated cells) and debulking by BNCT, is statistically significant. Further, nearly all of the immunoprophylaxis-treated surviving rats displayed long-term immunological memory, as they rejected contralateral i.c. rechallenge one year later (Table 2, groups 3 and 4). By contrast, of the rats that survived one year after BNCT treatment alone, only 1 out of 3 exhibited comparable immunological memory, which lends credence to our previous observation [35]. Group 2A is significantly different from groups 3 and 4 (Wilcoxon Two-Sample Test, $P^* < 0.02$).

Table 1 represents data pooled from four BNCT/immunoprophylaxis experiments (a total of 145 rats). Since there are slight variations in BPA delivery and tumor localization from experiment to experiment and rat to rat, it is difficult to adjust the BNCT dose to consistently achieve 50% survival. However in all four experiments, more than half of the rats that received adjunct immunoprophylaxis and that would probably have died had they received no treatment other than BNCT, actually did survive their brain tumors, which shows that the combination of BNCT plus immunoprophylaxis was more efficacious than either technique alone.

The i.c. 9LGS tumors that we treated on day 0 (14 days after inoculation) weighed approximately 40 mg. Ten million cells in culture represent approximately 3–6 mg protein as determined by the BCA protein assay method (Pierce, Rockford, Ill.) using bovine serum albumin as a standard. Therefore a 40 mg mass of 9LGS protein should represent no more than 13×10^7 cells. BNCT, at 40 Gy-Eq, allows about one clonogenic

tumor cell in 10,000 to survive [12]. By this estimation, there should be about 13,000 viable 9LGS cells left in the rat brain after BNCT. Therefore, immunization capable of rescuing 100% of rats that receive a 500,000 9LGS challenge s.c. and $> 50\%$ of rats that receive 5,000,000, 10,000,000 or 20,000,000 9LGS challenge s.c. is capable of rescuing only about 50% of rats with a minimal post-BNCT tumor burden. Evidently, there is a considerable discrepancy between the efficiencies of immune system-killing of tumor cells beneath the skin and in the brain. Innovations that increase the efficiency of i.c. tumor cell immune-based cytotoxicity, therefore, should improve the ability of the immune system to destroy tumor cells that remain after BNCT treatment.

We plan to apply what we have learned from the 9LGS model to treat less immunogenic and more invasive rat glioma tumor models, and ultimately to treat human GBM by initiating immunoprophylaxis as soon as possible after optimized neurosurgical and BNCT-based tumor debulking.

Conclusion

Immunoprophylaxis, by multiple s.c. injections of cultured, then irradiated autologous brain tumor cells after BNCT of the advanced brain tumor *in vivo*, is potentially an adjunct to therapy of human malignant astrocytomas that merits further experimental investigation in rats.

Acknowledgements

We wish to acknowledge the generous support of the Robert Leet and Clara Guthrie Patterson Trust, The Elsa U Pardee Foundation and the Institute of Pathology of the University of Bern to Dr. Henry M. Smilowitz. Dr. Jeffrey A. Coderre is supported by contract No. DE-AC02-98CH10886 from the office of Biological and Environmental Research of the U.S. Department of Energy.

References

1. Hartmann M, Jansen O, Egelhof T, Forsting M, Albert FK, Sartor K: Eifluss des Hirnodems auf das Rezidivwachstum maligner Gliome. Radiologe 38: 948–953, 1998
2. Slatkin DN: A history of boron capture therapy of brain tumors. Brain 114: 1609–1629, 1991
3. Barth RF, Soloway AH: Boron neutron capture therapy of brain tumors – current status and future prospects. Journal of Neuro-Oncology 33: 3–7, 1997
4. Coderre JA: Boron neutron-capture therapy. In: SA Leibel and TL Phillips (eds) Text Book of Radiation Oncology. W.B. Saunders, Philadelphia, pp 1263–1277, 1998
5. Coderre JA, Morris GM: The radiation biology of boron neutron capture therapy. Radiation Research 151: 1–18, 1999
6. Joel DD, Slatkin DN, Fairchild RG, Micca P, Nawrocky M: Pharmacokinetics and tissue distribution of the sulfhydryl boranes (monomer and dimer) in glioma-bearing rats. Strahlenther Onkol 165: 167–170, 1989
7. Joel DD, Fairchild RG, Laissue JA, Saraf SK, Kalef-Ezra JA, Slatkin DN: Boron neutron capture therapy of intracerebral rat gliosarcomas. Proc Natl Acad Sci USA 87: 9808–9812, 1990
8. Coderre JA, Glass JD, Fairchild RG, Micca PL, Fand I, Joel DD: Selective delivery of boron by the melanin precursor analogue p-boronophenylalanine to tumors other than melanoma. Cancer Research 50: 138–140, 1990
9. Miura M, Micca PL, Fisher CD, Heinricks JC, Donaldson JA, Finkel GC, Slatkin DN: Synthesis of a Ni tetracarboranylphenylporphyrin for BNCT: biodistribution and toxicity in tumor-bearing mice. Int J Cancer 68: 114–119, 1996
10. Fairchild RG, Saraf SK, Kalef-Ezra JA, Laster BH: Comparison of measured parameters from a 24 kev and a broad spectrum epithermal neutron beam for neutron capture therapy (NCT): an identification of consequential parameters. Medical Physics 17: 1045–1052, 1990
11. Coderre JA, Joel DD, Micca PL, Nawrocky MM, Slatkin DN: Control of intracerebral glioscarcomas in rats by boron neutron capture therapy with p-boronophenylalanine. Radiation Research 129: 290–296, 1992
12. Coderre JA, Makar MS, Micca PL, Nawrocky MM, Liu HB, Joel DD, Slatkin DN, Amols HI: Derivations of relative biological effectiveness for the high-LET radiations produced during boron neutron capture irradiations of the 9L rat glioscarcoma *in vitro* and *in vivo*. Int J Radiat Oncol Biol Phys 27: 1121–1129, 1993
13. Coderre JA, Button TM, Micca PL, Fisher C, Nawrocky MM, Liu AB: Neutron capture therapy of the 9L glioscarcoma using the p-boronphenylalanne-fructose complex. Int J Rad Onc Biol Phys 30: 643–52, 1994
14. Taylor JH, Goldhaber M: Detection of nuclear disintegration in a photographic emulsion. Nature, London 135: 341, 1935
15. Chanana AD: Boron neutron-capture therapy of glioblastoma multiforme at the Brookhaven Medical Research Reactor: A phase I/II study. FDA IND #43, 317, Protocol #4 Activated 5/10/96
16. Chanana AD, Capala J, Chadha M, Coderre JA, Diaz AZ, Elowitz EH, Iwai J, Joel DD, Liu HB, Ma R, Pendzick N, Peress NS, Shady MS, Slatkin DN, Tyson GW, Wielopolski L: Boron neutron capture therapy for glioblastoma multiforme: results from the initial phase I/II dose escalation studies. Neurosurgery: 44: 1182–1192, 1999
17. Coderre JA, Elowitz EH, Chadha M, Bergland R, Capala J, Joel DD, Liu HB, Slatkin DN, Chanana AD: Boron neutron-capture therapy for glioblastoma multiforme

using p-boronophenylalanine and epithermal neutrons: trial design and early clinical results. J Neuro-Oncology 33: 141–156, 1997
18. Ma R, Zhao X, Rarback HM, Yasumura S, Dilmanian FA, Moore RI, Lo Monte AF, Vodopia KA, Liu HB, Economos CD, Nelson ME, Alia JD, Vaswani AN, Weber DA, Pierson Jr RN, Joel DD: Calibration of the delayed-gamma neutron activation facility. Medical Physics 23(2): 273–277, 1996
19. Miura M, Micca PL, Fisher CD, Gordon CR, Heinrichs JC, Slatkin DN: Evaluation of carborane-containing porphyrins as tumor targeting agents for boron neutron capture therapy. The British Journal of Radiology 71: 773–781, 1998
20. Gabel D, Foster S, Fairchild RG: The Monte Carlo simulation of the biological effect of the $^{10}B(n, \alpha)^{7}Li$ reaction in cells and tissue and its implication for boron neutron capture therapy. Radiat Res 111: 14–25, 1987
21. Young HF, Merchant RE, Apuzzo MU: Immunocompetence of patients with malignant gliomas. In: Salcman M (ed) Neurobiology of Brain Tumors. Williams and Wilkins, Baltimore, 1991, pp 211–227
22. Grace Jr JT, Perese DM, Metzgar RS, Sasabe I, Hoidrige B: Tumor allograft responses in patients with glioblastoma multiforme. J Neurosurg 18: 159–167, 1961
23. Trouillas P, Lapras C: Immunotherapie active des tumeurs cérébrales: a propos de 20 cas. Neurochirurgie 16: 143–170, 1970
24. Febvre H, Maunoury R, Constans JP, Trouillas P: Delayed hypersensitivity reactions in patients bearing malignant brain tumors with human tumor cell lines grown *in vitro*. Int J Cancer 10: 221–232, 1972
25. Trouillas P: Immunologie et immunotherapie active des tumeurs cérébrales. Rev Neurol 128: 23–38, 1973
26. Mahaley Jr MS, Gillespie GY, Gillespie RP, Watkins PJ, Bigner DD, Wikstrand CJ, MacQueen JM, Sanfilippo F: Immunology of primary intracranial tumors. Part 8: Serological responses to active immunization of patients with anaplastic gliomas. J Neurosurg 59: 208–216, 1983
27. Dietric PY, Walker PR, Saas P, DeTribolet N: Immunobiology of gliomas: new perspectives for therapy. Ann NY Acad Sci 824: 124–140, 1997
28. Hodi FS, Dranoff G: Genetically modified tumor cell vaccines. Surg Oncol Clinics of N. America 7: 471–485, 1998
29. Schmidek HH, Neilsen SL, Schiller AL, Messer J: Morphological studies of rat brain tumors induced by N-nitrosomethylurea. J Neurosurg 34: 335–340, 1971
30. Russel DS, Rubinstein U: Pathology of Tumors of the Nervous System Williams and Wilkins, Baltimore, Fifth edn., 1989
31. Liu HB, Joel DD, Slatkin DN, Coderre JA: Improved apparatus for neutron capture therapy of rat brain tumors. Int J Radiat Oncol Biol Phys 28: 1167–1173, 1994
32. Rofstad EK, Brustad T: Tumor growth delay following single dose irradiation of human melanoma xenografts: correlations with tumor growth parameters, vascular structure, and cellular radiosensitivity. Br J Cancer 51, 201–210, 1985
33. Lentner C (ed), Geigy Scientific Tables, 8th edn., Vol.2. Geigy, Basle, 1982
34. Fleiss JL: Statistical Method for Ratios and Proportions, 2nd edn., John Wiley & Sons, NY, 1981
35. Smilowitz HM, Joel DD, Slatkin DN, Micca PL, Nawrocky MM, Youngs K, Coderre JA: Long-term immunological memory in the resistance of rats to transplanted intracerebral 9L gliosarcoma (9LGS) following subcutaneous immunization with 9LGS cells. Neuro-Oncol 46: 193–203, 1999
36. Tzeng J-J, Barth RF, Clendenon NR, Gordon WA: Adoptive immunotherapy of a rat glioma using lymphokine-activated killer cells and Interleukin 2. Cancer Research 50: 4338–4343, 1990
37. Holladay FP, Heitz T, Wood GW: Antitumor activity against established intracerebral gliomas exhibited by cytotoxic T lymphocytes, but not by lymphokine-activated killer cells. J Neurosurg 77: 757–762, 1992
38. Asai A, Miyagi Y, Hashimoto H, Lee SH, Mishima K, Sugiyama A, Tanaka H, Mochizuki T, Yasuda T, Kuchina Y: Modulation of tumor immunogenicity of rat glioma cells by s-Myc expression: eradication of rat gliomas *in vivo*. Cell Growth and Differentiation 5: 1153–1158, 1994
39. Barba D, Hardin J, Sadelain M, Gage FH: Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. Proc Natl Acad Sci USA 91: 4348–4352, 1994
40. Kruse CA, Schiltz PM, Bellgrau D, Kong Q, Kleinschmidt-DeMasters BK: Intracranial administrations of single or multiple source allogeneic cytotoxic T lymphocytes: chronic therapy for primary brain tumors. Journal of Neuro-Oncology 19: 161–168, 1994
41. Ram Z, Walbridge S, Heiss JD, Culver KW, Blaese RM, Oldfield EH: *In vivo* transfer of the human interleukin-2 gene: negative tumoricidal results in experimental brain tumors. J Neurosurg 80: 535–540, 1994
42. Perez-Cruet MJ, Trask TW, Chen S-H, Goodman JC, Woo SLC, Grossman RG, Shine HD: Adenovirus-mediated gene therapy of experimental gliomas, Journal of Neursci Res 39: 506–511, 1994
43. Fakhrai H, Dorigo O, Shawler DL, Lin H, Mercola D, Black KL, Royston I, Sobol RE: Eradication of established intracranial rat gliomas by transforming growth factor β antisense gene therapy. Proc Natl Acad Sci USA 93: 2909–2914, 1996
44. Siesjo PE Visse, Sjogren HO: Cure of established, intracerebral rat gliomas induced by therapeutic immunizations with tumor cells and purified APC or adjuvant IFN-γ treatment. Journal of Immunotherapy 19: 334–345, 1996
45. Kramm CM, Rainov NG, Sena-Esteges M, Barnett FH, Chase M, Herrlinger U, Pechan PA, Chiocca EA, Breakefield XO: Long-term survival in a rodent model of disseminated brain tumors by combined intrathecal delivery of herpes vectors and ganciclovir treatment. Human Gene Therapy 7: 1989–1994, 1996
46. Wakimoto H, Abe J, Isunoda R, Aoyagi M, Kirakawa K, Hamada H: Intensified antitumor immunity by a cancer vaccine that produces granulocyte-macrophage colony-stimulating factor plus interleukin 4. Cancer Res 56: 1828–1833, 1996

47. Rainov NG, Kramm CM, Aboody-Guterman K, Chase M, Ueki K, Louis DN, Harsh GR IV, Chiocca EA, Breakfefield XO: Retrovirus-mediated gene therapy of experimental brain neoplasms using the herpes simplex virus-thymidine kinase/ganciclovir paradigm. Cancer Gene Therapy 3: 99–106, 1996
48. Thompson RC, Pardoll DM, Jaffee EM, Ewend MG, Thomas MC, Tyler BM, Brem H: Systemic and local paracrine cytokine therapies using transduced tumor cells are synergistic in treating intracranial tumors. Journal of Immunotherapy 19(6): 405–413, 1997
49. Iwadate Y, Namba H, Tagawa M, Takenaga K, Sueyoshi K, Sakiyama S: Induction of acquired immunity in rats that have eliminated intracranial gliosarcoma cells by the expression of herpes simplex virus-thymidine kinase gene and ganciclovir administration. Oncology 54: 329–334, 1997
50. Ashley DM, Sampson JH, Archer GE, Batra SK, Bigner DD, Hale LP: A genetically modified allogeneic cellular vaccine generates MHC class I-restricted cytotoxic responses against tumor-associated antigens and protects against CNS tumors *in vivo*. J Neuroimmunology 78: 34–36, 1997
51. Plautz GE, Touhalisky JE, Shu S: Treatment of murine gliomas by adoptive transfer of *ex vivo* activated tumor-draining lymph node cells. Cellular Immunology 178: 101–107, 1997
52. Herrlinger U, Kramm CM, Johnston KM, Louis DN, Finkeistein D, Reznikoff G, Dranoff G, Breakefield XO, Yu JS: Vaccination for experimental gliomas using GM-CSF-transduced glioma cells. Cancer Gene Therapy 4: 345–352, 1997
53. Kruse CA, Roper MD, Kleinschmidt-DeMasters BK, Banuelos SJ, Smiley WR, Robbins JM, Burrows FJ: Purified herpes simplex thymidine kinase Retrovector^{TM} particles. I. *In vitro* characterization, *in situ* transduction efficiency, and histopathological analysis of gene therapy-treated brain tumors. Cancer Gene Therapy 4: 118–128, 1997
54. Glick RP, Lichtor T, Mogharbel A, Taylor CA, Cohen EP: Intracerebral versus subcutaneous immunization with allogeneic fibroblasts genetically engineered to secrete Interleukin-2 in the treatment of central nervous system glioma and melanoma, Neurosurgery 41: 898–907, 1997
55. Jean WC, Spellman SR, Wallenfreidman MA, Hall WA, Low WC: Interleukin-12-based immunotherapy against rat 9L glioma. Neurosurgery 42: 850–857, 1998
56. Wallenfriedman MA, Conrad JA, DelaBarre L, Graupman PCX, Lee G, Garwood M, Gregerson DS, Jean WC, Hall WA, Low WC: Effects of continuous localized infusion of granulocyte macrophage colony-stimulating factor and inoculations of irradiated glioma cells on tumor regression. J Neurosurg 90: 1064–1071, 1999
57. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre P-L, Cloughesy TF, Becker DP, Bronstein JM: Treatment of intracranial gliomas with bone marrow-derived dentritic cells pulsed with tumor antigens. J Neurosurg 90: 1115–1124, 1999
58. Ideda K, Ichikawa T, Wakimoto H, Silver JS, Deisboeck TS, Finkelstein D, Harsh IV GR, Louis DN, Bartus RT, Hochberg FH, Chiocca EA: Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. Nature (Medicine) 5: 881–887, 1999
59. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC: Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 90: 3539–3543, 1993
60. Brunda MJ, Gately MK: Antitumor activity of interleukin-12. Clin Immunol Immunopathol 71: 253–255, 1994

Address for offprints: H.M. Smilowitz, Department of Pharmacology, School of Medicine, University of Connecticut Health Center, 263 Farmington Ave., CT 06030-6125, USA; Tel.: 860-679-2710; Fax: 860-679-3693; E-mail: smilowitz@NSO1.UCHC.EDU