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Laboratory Investigation

Long-term immunological memory in the resistance of rats to transplanted intracerebral 9L gliosarcoma (9LGS) following subcutaneous immunization with 9LGS cells

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Comments

This paper reports on a series of experiments to study immunological memory in rats who had been vaccinated with the immunogenic 9L gliosarcoma cell line. Rats vaccinated by a subcutaneous injection of viable irradiated or non-irradiated cells or lysed cells, were challenged with a intracerebral (IC) injection of viable 9L cells. These experiments confirmed previous ones by other authors that most rats immunized with cells, irradiated or not, were protected from an IC challenge of 9L while the particulate cellular preparations offered no protection. Furthermore, the immunological response was specific and these animals remained protected as tumors failed to develop after a rechallenge with 9L in the contralateral hemisphere six months later. The immunological memory that resulted from the BNCT is an interesting, new finding which certainly requires more study. Together, this work provides further support to the approach of developing methods of immunoprophylaxis of weakly immunogenic cell lines and human brain tumors.

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Summary

Glioblastoma multiforme (GBM) is the most common primary human brain tumor. About 7000 new cases are diagnosed yearly in the USA. Despite current neurosurgical and postoperative radiotherapeutic tumor cytoreduction methods, in most cases occult foci of tumor cells infiltrate surrounding edematous brain tissues and cause recurrent disease within one year. GBM is almost invariably fatal within a few years after it is diagnosed. Our goal is to achieve long-term control of GBM by combining immunoprophylaxis with a radiation-based technique, such as boron neutron-capture therapy (BNCT), potentially capable of specifically targeting the infiltrating tumor cells while sparing the surrounding normal brain tissue.

It has long been known that the subcutaneous (sc) injection of irradiated cells or untreated cultured cells (and the removal of the resulting tumors) derived from the well characterized, highly immunogenic 9L gliosarcoma (9LGS) rat model into young isogenic rats can prevent tumor growth after subsequent sc or intracranial (ic) injection of untreated, otherwise lethal 9LGS cells. In this study we have confirmed, quantified and extended those findings to study the efficacy of such immunological memory in normal aging rats and in aging rats previously treated for ic 9LGS tumors by BNCT. (1) The sc injection of 5,000,000 untreated 9LGS cells and the surgical removal of the resulting tumors (method A) protected 80% of normal young rats from an ic challenge with 10,000 untreated 9LGS cells, and a single sc injection of 5,000,000 lethally X-irradiated 9LGS cells (method B) protected 66% of them, but multiple sc injections with a crude particulate fraction prepared from 9LGS cells were not protective. Protection is long-lasting since contralateral ic rechallenge of six-month survivors with an injection of 10,000 viable 9LGS cells resulted in 100% survival. (2) Normal one-year-old rats were only slightly less protected than were normal young rats, $\sim$70% rather than $\sim$80% (method A) and $\sim$60% rather than $\sim$66% (method B). (3) BNCT treatment alone resulted in partial immunological protection, as 30% of one-year post-BNCT survivors of ic 9LGS tumors

prevailed after contralateral ic rechallenge with 10,000 viable 9LGS cells. Moreover a single sc immunization with 5,000,000 untreated 9LGS cells prior to ic rechallenge boosted survival from 30% to 100%. The relevance of these observations to strategies of preclinical experimentation for immunoprophylaxis of malignant gliomas is discussed.

Introduction

Glioblastoma multiforme (GBM) is newly diagnosed in about 7000 US residents every year. It is the most common human primary brain tumor and it is almost always lethal; the median survival is about ten months. At the time of diagnosis, the tumor is usually large i.e. ~ 3 cm in greatest diameter. Generally, as much of the tumor as possible is removed surgically which alleviates imminent risk of death. Postoperative megavoltage photon radiotherapy delivered in doses approaching the limit of tolerance in normal brain structures (in effect, normal brain endothelial and oligodendroglial cells) 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, probably from occult microscopic foci of tumor cells infiltrating the surrounding edematous brain. Strategies being tested to improve survival include those that permit more effective irradiation of the tumor and reduced doses to normal brain structures i.e. the stereotactic implantation of radioactive 'seeds' and boron neutron-capture therapy (BNCT) [1–4] as well as tumor-directed chemotherapy, inhibition of tumor angiogenesis, immunotherapy [5,6] and techniques based on gene manipulation.

There exist a number of rat models for human malignant gliomas [7,8]. Among these are well-characterized cell lines that are weakly immunogenic, such as the RG2 [9,10] and the F98 [8] cell lines. There are also highly immunogenic cell lines such as the 9L gliosarcoma (9LGS) [11,12], which is thought to be identical in origin to the T9 rat glioma [8]. The 9LGS was initiated by the injection of N-nitrosomethylurea into the Fischer 344 rat; the F98 and RG2 cell lines each arose in the conceptus of pregnant CD Fischer rats injected with N-ethylnitrosourea. The 9LGS cell line has been extensively studied for nearly three decades. At Brookhaven National Laboratory (BNL), 10^4 viable 9LGS cells injected into the left or right striatum of a Fischer 344 rat consistently will grow to an approximately 40 mg intracranial tumor 14 days later [13] at which time it has several useful attributes as a therapeutic model of human gliomas. It is a rapidly growing, imminently lethal, unifocal, unilateral intracerebral malignancy, as are most human GBM tumors at the time of their initial diagnosis. They are not as difficult to eradicate in rats as is GBM in the human; however, untreated, the 9LGS intracerebral tumor is 100% lethal within 21 ± 3 (mean $\pm$ SD) days after its initiation from 10,000 implanted cells. Deaths from the intracranial (ic) 9LGS model [13] as well as from GBM [20] are caused by tumor growth and peritumoral cerebral swelling in most cases and craniospinal metastases *via* cerebrospinal fluid in the others. However microscopic brain infiltration is much more extensive from GBM than from the implanted 9LGS model.

Studies in the 1970s presented evidence that live or lethally-irradiated 9LGS cells injected subcutaneously (sc) into the flank of rats (immunizing injections) protected them from subsequent sc or ic challenge with autologous tumor cells (immunoprophylaxis) [14–16]. Analogous studies with the F98 and RG2 cell lines showed little or no protection [17]. In this paper we have confirmed the former findings and have extended them to study the efficacy of that immunological memory in aging rats. This investigation provides further impetus to develop immunoprophylaxis methods for weakly immunogenic cell lines in young and aging rats and thereafter to extend such methods to the injection of autologous, cultured, iatrogenically modified human glial tumor cells into patients in coordination with relatively-rapid tumor cytoreduction procedures that should result in minimal long-term perturbation of the immune system, such as operative neurosurgery, radiosurgery and BNCT.

Materials and methods

Cell lines and animals

The transplantable rat 9LGS gliosarcoma cell line was originally induced in a Fischer 344 rat by weekly iv injections of N-nitrosomethylurea [11] and subsequently serially passaged *in vitro* at the Department of Medicine, Montefiore Medical Center by Dr. Victor Hatcher before transfer to the late Dr. Ralph G. Fairchild at the Medical Department, BNL. Male Fischer 344 rats ($\sim$ 8–12 weeks old) were used for

most experiments. Control rats were rats chosen at random from the pool of normal-appearing experimental animals.

Cell culture

Cells were maintained at the University of Connecticut Health Center in DMEM medium (GIBCO) supplemented with inactivated 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 subcutaneous tumors, 9LGS cells were removed from T75 flasks. Cell clumps were mechanically disrupted using a Pasteur pipet; 5,000,000 cells were suspended in 0.1 ml of medium and injected sc in the left flank of a 0.054 mg/gbw ketamine/0.009 mg/gbw xylazine-anesthetized rat through a 25-gauge needle. Over the course of the 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 capacity glass injection vial and irradiated through a 2.5-cm diameter collimator aperture for 10 min with Cu/Al-filtered 100 kVp X-rays (6.60 Gy/min).

Surgical removal of subcutaneous 9LGS tumors

SC 9LGS tumors were excised aseptically 11–12 days after implantation. 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 $\sim 1\,cm^2$ on the surface. Approximately 75% of the tumors were mobile and operable; $\sim$ 15% of the tumors were fixed and too deep to remove; those rats were euthanized. The remaining $\sim$ 10% of the tumors had begun to invade the underlying muscle but were not too deep for excision.

Intracranial implantation of 9LGS cells

Tumors were initiated in ketamine/xylazine anesthetized male Fischer 344 rats (Taconic Farms, Germantown, NY) weighing about 200 g [13,18,19] by inoculating 1.0 μl of culture medium containing 10,000 cultured 9LGS cells into the left striatum 4–5 mm deep to a point on the serrated coronal suture 4 mm lateral to the midline and 1 mm anterior to the coronal plane containing the bregma. A 0.5-mm diameter burr hole was drilled at that point through the skull. A 27-gauge needle was fitted with a depth-limiting plastic collar to ensure cell injection 4–5 mm beneath the skull. Following a 30 sec infusion of the cells, an additional 30 sec was allowed for the cells to settle before removing the needle. This technique resulted in a locally expanding tumor with no evidence of blood- or CSF-borne metastases. Like human glioblastoma [20], this tumor can seed ventricular surfaces if injected slightly posterior to the targeted site (D.D. Joel, personal observations). Death ensues 21 ± 3 (mean $\pm$ SD) days after inoculation from an expanding supratentorial tumor around the site of inoculation and along the needle track [13].

Particulate fraction of GS-9L

9LGS cells were grown to confluence in T75 flasks. The cells in each flask were washed in phosphate-buffered saline (PBS), scraped into 20 mM Tris (pH 7.4) containing a protease inhibitor cocktail (0.5 mM PMSF, 2.5 μM Pepstatin, 2.5 μM leupeptin), disrupted in an ice-cooled hand-held Dounce homogenizer (40 strokes) and ultracentrifuged at $400{,}000\times\ g$ for 15 min in a TL100 Beckman ultracentrifuge. The pellets were resuspended in PBS; protein was assayed by the BCA method (Pierce, Rockford, IL).

Boron neutron-capture therapy

BPA-mediated boron neutron-capture therapy was performed as described by Coderre et al. [19]. The technique of slow-neutron irradiation was described by Joel et al. [13].

Measurements and statistics

Tumor volumes in mm^3 were calculated using orthogonal tumor dimensions in mm (width (w) and length (l)) according to $(w^2 l/2)$. A measure of the likelihood that there is a statistically significant difference between

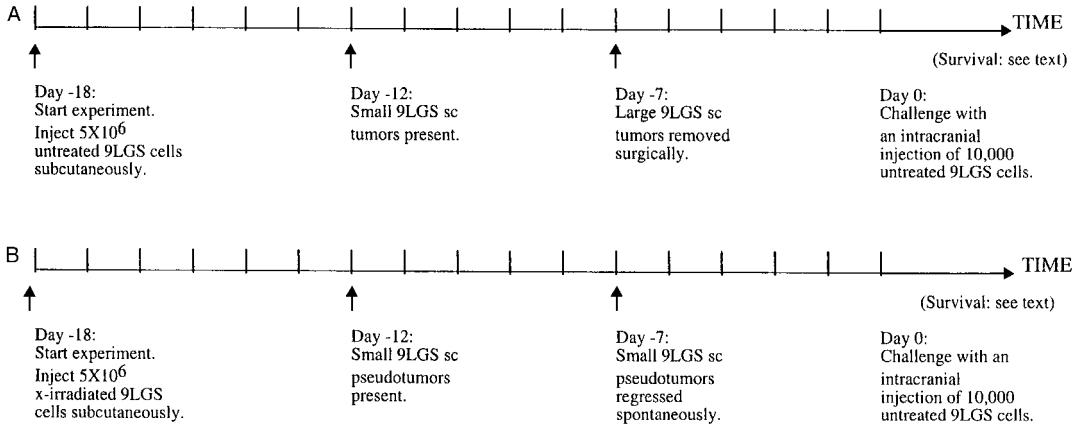

Figure 1. Experimental paradigms for 9LGS immunoprophylaxis: A: Experiment 1, untreated cells; B: Experiment 2, irradiated cells.

groups of animals, the confidence level p, was evaluated by the nonparametric Wilcoxon Two-Sample test with reference to published tables of acceptance regions for the corrected rank-sum for p values in the range of 0.1–0.001 [21]. p^* represents the confidence level for comparison with a group comprising the control group in the indicated experiment and the control group in a similar experiment.

Results

Experiment 1: resistance of normal young rats to the ic growth of 9LGS after excision of sc 9LGS tumors

Figure 1, line A illustrates the paradigm of experiment 1: Rats received sc injections of 5,000,000 9LGS cells in their left flanks on day −18. Six control rats were untreated. Eleven days later (day −7), the tumors that developed were excised surgically.

Table 1 shows the *in vivo* dimensions of the tumor on day −9, two days before their removal on day −7. Two of the 12 rats had to be euthanized; their tumors had become too large and they were inoperable. Of the 10 rats that did undergo surgery, no progressive tumor regrowth was seen in 9. On day 0, those 10 rats and six control rats were implanted with 10,000 9LGS cells ic. Of the 10 experimental rats, eight remained alive and tumor-free at six months when they were used for further experimentation. At six months these rats appeared normal with no wasting as would be expected if there were an autoimmune disease. Intracranial tumors grew in two of the ten rats. All six control rats died following concurrent ic implantation of 9LGS cells from the same preparation on days 20, 20, 20, 24, 24, 24 (median, 22 days). These experimental results are similar to those that had been obtained more than ten

Table 1. Resistance of normal young rats to the proliferation of ic-transplanted 9LGS cells (ic challenge) after surgical excision of sc 9LGS tumors

Rat #	Tumor volume (mm^3) (day −9)	Progressive sc tumor regrowth	Survival six months after ic 9LGS implantation
1	480	No	Yes
2	480	No	Yes
3	505	No	Yes
4	414	No	Yes
5	444	No	Yes
6	442	No	Yes
7	432	Yes***	No*
8	694	No	No**
9	335	No	Yes
10	466	No	Yes

Twelve male Fischer 344 rats received 0.1 ml sc injections containing 5,000,000 9LGS cells in the left thigh on day −18. Six control rats were untreated. Eleven days later (day −7) resultant tumors were excised from 10 of the rats (see Methods and Results); two of the rats were euthanized. On day 0, all rats were challenged with 10,000 9LGS cells ic (see Methods) and were followed for six months after ic challenge. The control rats died as follows: days 20, 20, 20, 24, 24, 24 (median, day 22).
Note: Death due to intracranial tumor regrowth was on day 27* and day 31** post contralateral ic 9LGS injection on day 0. ***Periosseous tumor not removed.

years earlier at BNL, using the same BNL strain of the rat 9LGS.

Experiment 2: protection of normal young rats from 9LGS cells injected ic by the prior sc injection of irradiated 9LGS cells

Figure 1, line B illustrates the paradigm of experiment 2. Rats received sc injections of 5,000,000 of the irradiated (66 Gy) 9LGS cells in their left flanks on day −18. Tumor-like swellings (pseudotumors $< 170\,mm^3$) were observed to form during the first week after cell injection and to regress spontaneously during the second week. Histologically, they are circumscribed zones of granulomatous inflammation. Putatively, the tumor-like swelling is caused in part by the influx of largely mononuclear leukocytic inflammatory cells attracted to the inoculum of irradiated but clonogenically disabled tumor cells.

Six control rats were untreated. On day 0, all twelve rats were implanted with 10,000 9LGS cells ic. Table 2 shows that, of the six experimental rats, four remained alive and tumor-free six months later when they were used for further experimentation. In contrast, all six control rats died following the ic implantation of 9LGS cells from the same preparation on days 20, 20, 20, 24, 24, 24 (median, 22) (same control group as for Experiment 1).

Table 2. Protection of normal young rats from 9LGS cells injected ic by the prior sc injection of irradiated 9LGS cells

Rat #	Survival at six months post-ic implantation of 9LGS cells
1	Yes
2	Yes
3	Euthanized, day 27**
4	Yes
5	Yes
6	Euthanized, day 28**

Six male Fischer 344 rats received sc injections of 5,000,000 irradiated 9LGS cells in their left flanks on day −18. Six control rats were untreated. On day 0, all the rats were implanted with 10,000 9LGS cells ic and were followed thereafter. All six control rats died following the ic implantation of 9LGS cells: days 20, 20, 20, 24, 24, 24 (median, 22) (same control group as for experiment 1).

**Due to ic tumor growth.

Experiment 3: the protection of normal young rats from 9LGS cells injected ic by the prior sc injection of irradiated 9LGS cells is specific and immunologically based

Experiment 3 also utilizes the paradigm of experiment 2 (Figure 1, line B). Table 3 shows that of the eight experimental rats that received a single sc injection of 5,000,000 irradiated 9LGS cells in their left flanks on day −18 and were implanted with 10,000 9LGS cells ic on day 0, five remain alive and tumor-free four months later (Group C). This 63% survival is similar to the 66% survival seen in experiment 2. In contrast, all seven control rats died at the expected time following the ic implantation of 9LGS cells from the same preparation (Group A). All eight immunized rats similarly died if they were challenged on day 0 with 10,000 D74 cells instead of 9LGS cells (Group D), as did their non-immunized controls (Group B). In contrast to Group C, the treatment of rats with 6.5 Gy whole body radiation one day prior to immunization on day −18 with a single injection of 5,000,000 irradiated 9LGS cells (Group E) resulted in death of all eight rats (median, 18.5 days).

Experiment 4: sc injections of particulate fraction prepared from 9LGS cells do not protect normal young rats from ic implanted 9LGS

Particulate fraction was prepared from 9LGS cells grown in cell culture. There were three groups of rats: Group 1, no injections ($N = 6$); Group 2, Incomplete Freund's Adjuvant (IFA) only ($N = 5$); Group 3, 9L membranes emulsified in IFA ($N = 5$); Group 3 rats received a series of five (biweekly) sc injections of membrane. Each rat received 400 μg membranes per injection in 800 μl, injected at four separate sites on the back; 200 μl each (injections #1) and 800 μg membrane per injection in 800 μl, injected at four separate sites on the back in 200 μl at each site (injections #2–5). 10,000 viable 9LGS cells were injected ic between the third and fourth sc injections. There were no statistically meaningful differences in growth among these three groups of transplanted tumors:

Group 1, controls (no injections); $N = 6$. Survival in days: 17, 18, 18, 19, 20, 28 (median, 18.5). Group 2, IFA only; $N = 5$. Survival in days: 19, 19, 20, 24, 24 (median, 20). Group 3, 9LGS membranes emulsified

Table 3. The protection of normal young rats from 9LGS cells injected ic by the prior sc injection of irradiated 9LGS cells is specific and immunologically based

Group	*N*	Surviving fraction	Death (days)	Median
A: Not treated and challenged ic with 10,000 9LGS cells	7	0/7	17, 17, 20, 20, 21	20
B: Not treated and challenged ic with 10,000 D74(RG2) cells	6	0/6	19, 20, 21, 22, 22, 23	21.5
C: Pre-treated with a single injection of 5,000,000 irradiated 9LGS cells (day −18) and challenged with 10,000 9LGS cells ic	8	5/8*	20, 25, 53	25
D: Pre-treated with a single injection of 5,000,000 irradiated 9LGS cells (day −18) and challenged with 10,000 D74 cells ic	8	0/8	19, 19, 20, 20, 20, 20, 21, 21	20
E: Treated with 6.5 Gy whole body gamma radiation one day prior to pre-treatment with a single injection of 5,000,000 irradiated 9LGS cells (day −18) and challenged with 10,000 9LGS cells ic	8	0/8	17, 18, 18, 18, 19, 20, 20, 20	18.5

*Surviving rats euthanized after four months.

in IFA; $N = 5$. Survival in days: 17, 18, 18, 18, 21 (median, 18).

Experiment 5: protection of six-month-old survivors (Table 1) from contralateral ic injections of 9LGS (ic rechallenge): evidence for long-term maintenance of anti-9LGS immune competence

The eight surviving rats (Table 1) and the four surviving rats (Table 2) that survived the implantation of 10,000 9LGS cells ic on the left side were rechallenged after six months with 10,000 GS9L cells implanted ic on the contralateral (right) side. Table 4, Group 1 shows that five of the Table 1 rats survived the ic cell re-implantation procedure; the other three rats died accidentally from inappropriate anesthesia. Of the five survivors that were successfully re-implanted with 9LGS cells ic, all five lived at least eight months post ic rechallenge; none died of brain tumors. Table 4, Group 2 shows that, of the four surviving rats in Table 2 rechallenged with 10,000 9LGS cells ic on the contralateral side, all four lived at least 11 months after the rechallenge; none died of brain tumors. Table 4, Group 3 shows that all six rats of the control group died of their ic cell implants (days 21, 23, 23, 24, 24, 27; median, 23.5).

Table 4. Protection of six-month-old survivors of ic 9LGS (Table 1) from contralateral ic injections of 9LGS (ic rechallenge): evidence suggesting the long-term maintenance of anti-9LGS immune competence

Group	Survivors*
1. Treated (Table 1) and rechallenged ic	5/5
2. Treated (Table 2) and rechallenged ic	4/4
3. Not treated and challenged ic	0/6
4. One-year-old normal rats, untreated and challenged ic	0/6 (Table 6, Group A)

The eight surviving rats (Table 1) and the four surviving rats (Table 2) that survived the implantation of 10,000 9LGS cells ic on the left side, were rechallenged after six months with 10,000 9LGS cells implanted ic on the contralateral (right) side. Group 1: five of the Table 1 rats that survived the ic cell re-implantation procedure. Group 2: the four surviving rats in Table 2 that were successfully rechallenged with 10,000 9LGS cells ic on the contralateral side. Group 3: All six rats of the control group died of concurrently ic-implanted 10,000 9LGS cells: days 21, 23, 23, 24, 24, 27; median, 23.5. Group 1 vs. Group 3, $p < 0.05$; Group 2 vs. Group 3, $p < 0.02$. Group 1 survivors: euthanized on days 261, 348, 365, 365, 365. Group 2 survivors: euthanized on days 348, 365, 365, 365. None of the euthanized rats had brain tumors.

*Followed for twelve months after ic rechallenge with 10,000 9LGS cells.

Experiment 6A: protection of one-year-old BNCT survivors of ic 9LGS from contralateral ic injections of 9LGS: A. Evidence suggesting persistent anti-9LGS immune competence in aged rats

Ten thousand 9LGS cells were implanted ic in rats on day 0. On day 14, when the brain tumors weighed approximately 40 mg, the rats were treated by boron neutron-capture therapy (Coderre et al., 1993). 100% of control (untreated) rats died (median survival post 9LGS cell inoculation was 21 ± 3 days). One year later, six of the surviving BNCT-treated rats were injected with 10,000 9LGS cells ic on the contralateral side. Table 5, Group A shows that four of the six rechallenged rats died of brain tumors (survival in days 16, 17, 22, 23; median, 19.5). Of the two rats that survived the ic rechallenge, one had to be euthanized at day 90 due to a large abscess in the left eye; no brain tumor was detected at necropsy. The remaining rat died at day 153; no brain tumor was found at necropsy.

Six age-matched, one-year-old control rats were challenged with 10,000 9LGS cells ic at the same time as the BNCT rechallenge. Of the six rats that received 10,000 9LGS cells ic, all died (days 17, 17, 18, 19, 20, 27; median, 18.5); Table 5, Group C.

In a similar but separate experiment, nine rats that had received 10,000 9LGS cells ic, were treated by BNCT 14 days later and had survived one year were pooled from various experiments and rechallenged with 10,000 9LGS cells ic on the contralateral side. Six of the nine rechallenged rats died of progressively growing brain tumors (days 20, 21, 21, 29, 34, 66). However, three rats survived the ic rechallenge and died on day 132 or were euthanized on days 176 and 237. No brain tumors were found in these rats at necropsy. From the pooled data, Table 5, Group A and these nine rats, we conclude that one-year-old BNCT survivors exhibit anti-tumor immunologic memory ($p^* < 0.005$).

Experiment 6B: protection of one-year post-BNCT survivors of ic 9LGS from contralateral ic growth of 9LGS cells by prior sc injections of irradiated 9LGS cells

Five rats that were one year BNCT survivors (as above) received sc injections of 5,000,000 irradiated 9LGS cells in their right flanks 18 days prior to rechallenge with 10,000 9LGS cells ic on the contralateral side.

Table 5. Protection of one-year-old rat BNCT survivors from ic injections of 9LGS: evidence suggesting the persistence of some anti-9LGS immune competence with age

Group A*:	One-year-old post-BNCT survivors rechallenged ic with 10,000 9LGS cells ic; $N = 6.\ 2/6$ surviving; (days 16, 17, 22, 23) (median, day 19.5)
Group B:	One-year-old post-BNCT survivors that received a sc injection of 5,000,000 irradiated 9LGS cells prior to ic rechallenge with 10,000 9LGS cells ic; $N = 5$. (5/5 surviving; euthanized on days 135, 150, 150, 161, 203. None of the euthanized rats had brain tumors)
Group C:	One-year-old naive rats (no previous treatments); $N = 6$ (days 17, 17, 18, 19, 20, 27) (median, day 18.5)

10,000 9LGS cells were implanted ic in male Fischer 344 rats on day 0. On day 14, when the brain tumors weighed ~ 40 mg, the tumors were treated by boron neutron-capture therapy (13, 19). 100% of the untreated control rats in this group died (median survival post 9LGS cell innoculation was 21 ± 2 days). Group A: six of the surviving rats were injected with 10,000 9LGS cells ic on the contralateral side one year later. Group B: five, one-year BNCT survivors received sc injections of 5,000,000 irradiated 9LGS cells in their right flanks 18 days prior to rechallenge with 10,000 9LGS cells ic in the contralateral side. The irradiated cells formed small tumors which spontaneously regressed. On day 0, the five rats in this group were injected with 10,000 9LGS cells ic. Group C: six naive control rats, also one year old, were challenged with 10,000 9LGS cells ic at the same time as the BNCT rechallenge. Group A *versus* Group C, $p^* < 0.05$; Group B *versus* Group C, $p < 0.01$; Group A *versus* Group B, $p > 0.1$.

**Note*: One of the two surviving rats was euthanized on day 90 after ic rechallenge due to a left eye infection. No ocular or brain tumor was found at necropsy. The other surviving rat died on day 153. No brain tumor was found at necropsy.

The irradiated cells formed small pseudotumors, which regressed spontaneously within two weeks. On day 0, the five rats in this group and the six aged, naive controls (Table 5, Group C) were injected with 10,000 9LGS cells ic. Table 5, Group B shows that all the one-year BNCT survivors that received sc injections of irradiated cells prior to ic rechallenge survived at least four months; none of these rats died of brain tumors while all the untreated controls (Table 5, Group C) died of brain tumors.

Experiment 7: protection of normal one-year-old rats from 9LGS cells injected ic by the prior subcutaneous injection of 9LGS cells

Seven one-year-old rats were injected sc with 5,000,000 viable 9LGS cells. The resultant tumors were removed after eleven days. One week later the rats were challenged with 10,000 9LGS cells injected ic.

Table 6. Protection of one-year-old rats from 9LGS cells injected ic by the prior sc injection of 9LGS cells

Group A:	One-year-old naive controls (no previous treatments); $N = 6$; 0/6 survivors. (days 17, 17, 18, 19, 20, 27) (median, day 18.5)
Group B:	One-year-old rats injected sc with 5,000,000 viable 9LGS cells. The resultant tumors were removed 11 days later, prior to challenge with 10,000 9LGS cells ic; $N = 7$; 5/7 survived for greater than nine months and were euthanized on days 286, 342, 365, 365, 365. None of these rats had brain tumors. 2/7, survival in days: 23, 44.
Group C:	One-year-old rats injected sc once with 5,000,000 X-irradiated 9LGS cells prior to challenge with 10,000 9LGS cells ic; $N = 5$; 3/5, survived for greater than nine months and were euthanized on days 278, 365, 365. None of these rats had brain tumors. 2/5, survival in days: 23, 30.

Group A: one-year-old naive rats (controls). Group B: seven, one-year-old rats were injected sc with 5,000,000 viable 9LGS cells. The resultant tumors were removed after 11 days. One week later the rats were challenged with 10,000 9LGS cells injected ic. Group C: five, one-year-old rats were injected sc with 5,000,000 X-irradiated 9LGS cells. Eighteen days later the rats were challenged with 10,000 9LGS cells injected ic. Group A *versus* Group B, $p^* < 0.001$; Group A *versus* Group C, $p < 0.02$; Group B *versus* Group C, $p > 0.1$.

Table 6, Group B shows that, of the seven aged rats so treated, five have survived over nine months. Similarly, five one-year-old rats were injected sc with 5,000,000 X-irradiated 9LGS cells. Eighteen days later the rats were challenged with 10,000 9LGS cells injected ic. Table 6, Group C shows that, of the five aged rats so treated, three have survived over nine months. All six of the one-year-old naive (control) rats succumbed to brain tumors induced by the injection of 10,000 9LGS cells ic; Table 6, Group A.

Discussion

The immunogenicity of chemically induced murine tumors was originally shown in the 1950s. When an established tumor was excised surgically the host remained resistant to subsequent implants of cells from that tumor but not from other tumors [22,23]. Such specific immunogenicity could also be induced by the injection of irradiated tumor cells [24] or subtumorigenic numbers of replicating tumor cells [25]. By those criteria, the 9LGS tumor was deemed immunogenic 3–4 decades ago.

Immunoprophylaxis studies in the mid seventies [14,15] and more recently [37] showed that whereas challenge with as few as 5000 9LGS cells sc or 1000 cells ic resulted in progressive tumor growth in all animals tested, prior immunization with irradiated 9LGS cells protected rats from subsequent sc or ic challenge. Similarly, our results show that a single sc injection of 5,000,000 X-irradiated (50 Gy) 9LGS cells 18 days prior to the challenge of 10,000 viable 9LGS cells increases the number of rats that survive from 0/6 (control) to 4/6, although the 66% survival rate is lower than the 90% and 100% survival rates, respectively, reported by Blume and Denlinger. Improved survival rates were obtained when unirradiated 9LGS cells were used for immunization. Untreated 9LGS cells (5,000,000) injected sc produce progressively growing, non-metastasizing tumors weighing 580 to 1270 mg (mean $= 870 \pm 200$ g) by eleven days. Those were removed surgically; 80% of those rats survived a challenge with 10,000 unirradiated 9LGS cells injected ic seven days later. These results suggest that the sc injection of 5,000,000 untreated 9LGS cells and the removal of the resulting tumors 11–12 days later produces a more protective immune response to ic challenge than does a single sc injection of 5,000,000 X-irradiated cells. Injection of untreated tumor cells and surgical removal of the resulting tumors was also used by Geyer and Landay [26] in their sc immunoprophylaxis experiments.

Our sc injection of a crude particulate fraction prepared from sister cultures of 9LGS cells, however, did not protect rats from ic challenge; rats that received a series of five injections of emulsified IFA in PBS or 9LGS crude particulate fraction (800 μg particulate fraction per injection) in PBS emulsified in IFA all died as did the untreated control rats; there was no statistical difference between these groups. Injections of 0.4 to 12.0 mg of soluble 9LGS tumor extract also failed to confer tumor immunity [27]. However the immunization conditions used to inject particulate fraction or soluble extract were not optimized in our studies. The recent report by Liau [51] that dendritic cells pulsed with acid-extracted peptides from 9L cells could confer immunity to autologous tumor challenge suggests that further research on subcellular tumor immunogens is warrented.

Many issues have been raised about the potential usefulness of immunoprophylaxis in human glioma therapy. Since the median age at which GBM arises in Europe and North America is about 60 years, any

extensively useful immunoprophylaxis for GBM recurrence should be relevant to old as well as young patients. Further, any useful immunoprophylaxis must afford long-term protection. Using the highly immunogenic 9LGS model, we have shown that one-year-old rats can be protected by immunization with untreated or X-irradiated 9LGS cells (Table 5). Of seven such rats immunized with untreated 9LGS cells prior to ic challenge, five have survived > six months ($\sim$ 70%); of five such rats immunized with 5,000,000 X-irradiated 9LGS cells, three have survived (60%). These survival rates are slightly lower than, but not statistically different from the corresponding rates obtained with the younger rats (80–100% and 66%, respectively). Immune system function and the ability to respond to a variety of antigens decreases with age [28–30]. An age-determined decline in the ability to respond to rejection antigens on syngeneic tumors has been documented [31–33]. An age-dependent decline in the ability of mice to repond to some forms of immunotherapy has also been documented [34]. Therefore we postulated that aged rats would be less capable than young rats in responding to the immunoprophylaxis described. However, we observed no such age-dependent effect that was statistically significant although we did not so test senescent rats older than 15 months [35].

Our experimental results are consistent with a role for immunological memory up to at least one year: (1) Rats pretreated with X-irradiated 9LGS cells or untreated 9LGS cells that have survived six months after ic challenge (Tables 1 and 2) have all survived a second 9LGS ic challenge on the contralateral side (ic rechallenge). All of the control rats died between days 21 and 27 after ic challenge ($p < 0.02$; $p < 0.05$, respectively; $p^* < 0.005$; $p^* < 0.001$, respectively). (2) Rats challenged with 9LGS cells ic and successfully treated by BNCT (one-year BNCT survivors) are partially protected when rechallenged with 9LGS cells ic on the contralateral side; two of six (33%) one-year BNCT surviving rats have survived for $>$ 4 months ($p^* < 0.05$). Moreover, when such BNCT-treated one-year survivors are boosted by a single sc injection of 5,000,000 lethally X-irradiated 9LGS cells prior to ic rechallenge on the contralateral side, five of five (100%) rats have survived > four months. This can be compared with the 60% survival rate of the one-year-old naive rats preinjected with X-irradiated cells and the 33% survival rate of unboosted one-year BNCT survivors. These results are consistent with the notion that rats treated for established 9LGS tumors 14 days after the ic injection of 10,000 viable cells by BNCT possess partial immunological memory as much as one year later; antigens from ic 9LGS tumors are accessible to the cells of the immune system. Furthermore, all rats boosted with 5,000,000 irradiated cells sc became resistant to another ic challenge. Two recent studies have shown immunological memory in their systems but only up to 2–3 months [36,37]. Investigations throughout the 70s and early 80s established that immunity to syngeneic tumor rechallenge due to prior immunization with irradiated or untreated tumor cells is mediated by T cells [38–42]. This has partially explained how tumors that possess transplantation rejection antigens escape destruction by the host immune system when lymphocytes are overwhelmed by tumor mass and how prior immunization coupled to effective debulking may provide short or long-term resistance to tumor regrowth. However for therapy, studies on the nature of immunogens and of immunization protocols are needed to optimize the balance between lymphocyte subtypes involved in tumor promotion and tumor rejection [43]. For example, a course of multiple injections in several different fields of lymphatic drainage with irradiated 9LGS cells might prove more effective than a single injection; appropriately genetically modified tumor cells might prove better immunogens [44].

Given the virtual inevitability of the recurrence of human GBM, usually 6–9 months after initial surgical debulking and radiotherapy, a course of immunoprophylaxis initiated shortly after these initial therapies could prove beneficial. Such immunoprophylaxis could involve the sc injection of live GBM cells and the subsequent removal of the resulting tumors, since GBM cells are known not to metastasize, the injection of irradiated GBM cells, or the injection of subcellular tumor immunogens. This paper is a study of immunoprophylaxis in a rat tumor model of known high immunogenicity. It is our intention that these studies may serve as a frame of reference for subsequent studies of immunoprophylaxis in rat glioma models of low immunogenicity, which we believe could lead to clinical investigations of immunoprophylaxis to avert recurrence of palliatively treated human malignant astrocytomas. Human gliomas are generally considered to be of low immunogenicity, although, to our knowledge, the range of their immunogenicities has never been quantified. There have been several reports of active immunotherapy of human gliomas with and without standard radiotherapy (for review, see Young

et al. [45]) that were well tolerated and resulted in positive delayed hypersensitivity reactions [46–50]; some provided modest extensions of life. New methods of radiotherapy, such as BNCT, which are implemented in several days rather than in many weeks, are less damaging than standard radiotherapy to normal brain parenchyma and blood vessels and may better preserve the access of immune cells to tumors. Recent advances in the cell and molecular biology of immunity justify intensive study of post-radiotherapy immunoprophylaxis for GBM.

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