Molecular Targeting and Treatment of EGFRvIII-Positive Gliomas Using Boronated Monoclonal Antibody L8A4

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Abstract

Purpose: The purpose of the present study was to evaluate a boronated EGFRvIII-specific monoclonal antibody, L8A4, for boron neutron capture therapy (BNCT) of the receptor-positive rat glioma, F98$_{npEGFRvIII}$.

Experimental Design: A heavily boronated polyamido amine (PAMAM) dendrimer (BD) was chemically linked to L8A4 by two heterobifunctional reagents, *N*-succinimidyl 3-(2-pyridyldithio)propionate and *N*-(*k*-maleimidoundecanoic acid)hydrazide. For *in vivo* studies, F98 wild-type receptor-negative or EGFRvIII human gene-transfected receptor-positive F98$_{npEGFRvIII}$ glioma cells were implanted i.c. into the brains of Fischer rats. Biodistribution studies were initiated 14 days later. Animals received [^{125}I]BD-L8A4 by either convection enhanced delivery (CED) or direct i.t. injection and were euthanized 6, 12, 24, or 48 hours later.

Results: At 6 hours, equivalent amounts of the bioconjugate were detected in receptor-positive and receptor-negative tumors, but by 24 hours the amounts retained by receptor-positive gliomas were 60.1% following CED and 43.7% following i.t. injection compared with 14.6% ID/g by receptor-negative tumors. Boron concentrations in normal brain, blood, liver, kidneys, and spleen all were at nondetectable levels (<0.5 µg/g) at the corresponding times. Based on these favorable biodistribution data, BNCT studies were initiated at the Massachusetts Institute of Technology Research Reactor-II. Rats received BD-L8A4 (~ 40 µg ^{10}B/~ 750 µg protein) by CED either alone or in combination with i.v. boronophenylalanine (BPA; 500 mg/kg). BNCT was carried out 24 hours after administration of the bioconjugate and 2.5 hours after i.v. injection of BPA for those animals that received both agents. Rats that received BD-L8A4 by CED in combination with i.v. BPA had a mean ± SE survival time of 85.5 ± 15.5 days with 20% long-term survivors (>6 months) and those that received BD-L8A4 alone had a mean ± SE survival time of 70.4 ± 11.1 days with 10% long-term survivors compared with 40.1 ± 2.2 days for i.v. BPA and 30.3 ± 1.6 and 26.3 ± 1.1 days for irradiated and untreated controls, respectively.

Conclusions: These data convincingly show the therapeutic efficacy of molecular targeting of EGFRvIII using either boronated monoclonal antibody L8A4 alone or in combination with BPA and should provide a platform for the future development of combinations of high and low molecular weight delivery agents for BNCT of brain tumors.

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Boron neutron capture therapy (BNCT) is based on the selective delivery of ^{10}B to a tumor followed by neutron irradiation. The resulting nuclear capture and fission reactions (^{10}B + n → [^{11}B] → ^{4}He + ^{7}Li) yield α-particles (^{4}He) and recoiling ^{7}Li nuclei. Administration of the boron delivery agent(s) and neutron irradiation can be manipulated independently, so that the interval between them can be optimized to give the greatest tumor-to-normal tissue boron concentration ratios. For BNCT to be successful, there must be a sufficient amount of ^{10}B in the tumor, low levels in blood and normal tissues in the field of irradiation, and enough low-energy thermal neutrons must be delivered to the tumor site. The major challenge for effectively treating high-grade gliomas with BNCT is the delivery of sufficient amounts of ^{10}B and neutrons to individual tumor cells to sustain a lethal ^{10}B(n,α)^{7}Li capture reaction. Two low molecular weight boron delivery agents, sodium borocaptate (BSH) and boronophenylalanine (BPA), have been used in both experimental animal and clinical

studies. Several recent reviews and monographs are available for those readers interested in more detailed information relating to these studies and BNCT in general (1–3).

The gene encoding epidermal growth factor receptor (EGFR) often is amplified or mutated in human gliomas (4), but its expression is low or undetectable in normal brain (5). Based on this, EGFR and its mutant isoform, EGFRvIII, are under intensive investigation as molecular targets for the specific delivery of diagnostic and therapeutic agents to brain tumors (6–8). We have been interested in the possibility of using either EGF itself (9–13) or anti-EGFR monoclonal antibodies (mAb; refs. 14, 15) as boron delivery agents for NCT of high-grade gliomas. To fully evaluate this approach, we initially produced a series of transfectants of the F98 rat glioma, which expressed wild-type human EGFR (11, 12). Using the $F98_{EGFR}$ glioma model, we evaluated boronated EGF as a delivery agent for NCT and established proof-of-principle that there was a significant therapeutic response following BNCT (13). However, although wild-type EGFR is overexpressed in a variety of human malignancies, it also is expressed in normal liver and spleen, which can take up significant amounts of either anti-EGFR mAb (16) or EGF ligand (17–19) following systemic administration. On the other hand, EGFRvIII, which has an in-frame deletion of exons 2 to 7 of the extracellular domain (7, 20), is expressed and amplified in up to 54% of human glioblastomas and 75% of anaplastic astrocytomas (21). Because EGFRvIII seems to be a truly tumor-specific target, several mAbs directed against this receptor have been produced for diagnostic and therapeutic purposes (6, 22). These include DH8.3 (23), 806 (24–26), and, the subject of the present report, L8A4 (27–31).

We recently have described an EGFRvIII-expressing variant of the F98 rat glioma, designated $F98_{npEGFRvIII}$, which is syngeneic to Fischer rats. This was produced by transfecting wild-type F98 glioma cells with the gene encoding human EGFRvIII, and one of the transfectants stably expressed a mutant, nonphosphorylated form of the protein. This tumor model recently has been described by us in detail elsewhere (31, 32). Using this model, we evaluated the biodistribution of [^{125}I]L8A4 in $F98_{npEGFRvIII}$ glioma-bearing rats following i.v. or i.t. injection or convection enhanced delivery (CED) and showed specific molecular targeting of the receptor (31). In the present study, we initially evaluated the biodistribution of boronated L8A4 in both non-tumor-bearing and $F98_{npEGFRvIII}$ glioma-bearing rats. Based on favorable biodistribution data, we then initiated therapy studies using BD-L8A4, given by either i.t. injection or CED, alone or in combination with i.v. BPA. As described in detail in this report, CED of BD-L8A4 followed by BNCT resulted in a significant prolongation in the survival times of $F98_{npEGFRvIII}$ glioma-bearing rats with a subset of cured animals.

Materials and Methods

Preparation and purification of boronated L8A4 bioconjugate. Either fourth- or fifth-generation polyamido amine (PAMAM) dendrimers (Sigma, St. Louis, MO) were boronated with the methylisocyanato polyhedral borane anion $Na(CH_3)_3NB_{10}H_8NCO$, which was synthesized by us, to yield a boronated dendrimer (BD) using a procedure described previously by us (15). The BD was reacted with N-succinimidyl 3-(2-pyridyldithio)propionate (Pierce Chemical Corp., Rockford, IL) and the resulting product was cleaved with DTT to yield a SH-containing BD. mAb L8A4 was derivatized at the Fc region with the

heterobifunctional reagent N-(k-maleimidoundecanoic acid)hydrazide (Pierce) and then linked to the sulfhydryl-containing BD to yield the bioconjugate BD-L8A4 (15). This was purified by column chromatography using a Sephadex-G50 column (Amersham Pharmacia Biotech, Piscataway, NJ) and eluted with 0.1 mol/L Tris and 0.2 mol/L NaCl buffer (pH 8.5). Fractions (1 mL) were collected and protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm using a Beckman DU-6 spectrophotometer (Beckman Instruments, Inc., Irvine CA). Boron was quantified by direct current plasma-atomic emission spectroscopy using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA) as described previously (33). Fractions containing the highest concentrations of both protein and boron were pooled and used in the studies described in the following section.

Radioiodination of BD-L8A4. BD-L8A4 bioconjugates were reacted with Bolton-Hunter reagent (Pierce) to introduce an aromatic ring for radioiodination. A 10-fold molar excess of Bolton-Hunter reagent was added to BD-L8A4 and cooled on ice for 1 hour following which unreacted reagent was removed using a Bio-Spin P-6 column (Bio-Rad Laboratories, Hercules, CA). BD-L8A4 and BD then were radioiodinated with [^{125}I]NaI by the procedure described by us (12) using chloramine-T (ICN Biomedicals, Inc., Costa Mesa, CA) at a concentration of 2 mg/mL in 0.5 mol/L phosphate buffer (pH 7.5). [^{125}I]BD-L8A4 was shown to be stable and was not dehalogenated for at least 1 week when kept at 4°C.

In vitro receptor-binding assay. The F98 glioma cell line (CRL-2397, American Type Culture Collection, Manassas, VA) was derived from a CD-Fischer rat whose mother had received N-ethyl-N-nitrosourea during pregnancy and it has been described in detail elsewhere (34). F98 wild-type ($F98_{WT}$) cells and $F98_{npEGFRvIII}$ cells (31, 32) were used in the studies described in the following sections. All cells were grown in DMEM containing glucose, L-glutamine, and 10% fetal bovine serum. Receptor-binding activity of ^{125}I-labeled, boronated, and native L8A4 with $F98_{npEGFRvIII}$ cells was studied by a competitive binding assay. $F98_{npEGFRvIII}$ cells ($\sim 3 \times 10^4$ per well) were seeded into 24-well flat-bottomed plates (Corning, Inc., Corning, NY) and allowed to attach overnight. Medium was replaced with DMEM containing 10% fetal bovine serum and 1 μmol/L dexamethasone (Sigma) and incubated overnight at 37°C. This was replaced with serum-free DMEM and incubated for 2 hours following which the cells were washed with PBS (pH 7.2). Varying concentrations of native and boronated L8A4 were added to triplicate wells and incubated at ambient temperature in an atmosphere containing 5% CO_2 for 2 hours. The cells then were washed, and medium containing [^{125}I]L8A4 at a concentration of 0.18 nmol/L was added. They were incubated for an additional 2 hours at ambient temperature, washed three times with PBS, and harvested using 0.5 mmol/L EDTA in PBS, and cell-associated radioactivity was determined by γ-scintillation counting (model 1185, Tm Analytic, Elk Grove Village, IL). $F98_{WT}$ and $F98_{npEGFRvIII}$ cells were seeded into T-150 flasks and cultured for 3 days to establish a confluent monolayer. Cells were washed twice with PBS and then harvested by incubating them with 0.5 mmol/L EDTA at 37°C for 10 minutes. Following removal of EDTA, 10^6 cells were added to 1.5 mL tubes to which varying amounts of [^{125}I]L8A4 in 0.1% bovine serum albumin had been added, and these were incubated at 4°C for 90 minutes. The cells then were washed three times with PBS, cell-bound and free radiolabeled antibody or bioconjugate were separated by centrifugation, and radioactivity was quantified by γ-scintillation counting. The K_a and the number of receptor sites were derived by nonlinear regression analysis.

Tumor implantation and CED of BD-L8A4. All animals studies were done in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and the protocol was approved by the Institutional Laboratory Care and Use Committee of The Ohio State University (Columbus, OH). CD-Fischer rats (Charles River Laboratories, Wilmington, MA), weighing 200 to 220 g, were anesthetized with a 1.2:1 mixture of ketamine/xylazine. Following this, tumor cells were implanted stereotactically as described

previously (35). A small plastic screw (Arrow Machine Manufacturing, Inc., Richmond, VA) with an entry port, which allowed insertion of a 27-gauge needle, was embedded into the calvarium before implantation. For CED, a plastic cannula was inserted into the entry port and then advanced 5 mm below the dura into the right caudate nucleus of non-tumor-bearing animals or into the tumor of glioma-bearing rats. CED of BD-L8A4 was carried out using a syringe pump (Harvard Apparatus Co., Cambridge, MA) as described previously by us (12). The bioconjugate was diluted with PBS to yield a concentration of 5 μCi/50 μg BD-L8A4/10 μL. Evans blue dye (3 μL) was added to every 100 μL [^{125}I]BD-L8A4 solution, so that the site of infusion subsequently could be visualized grossly within the brain parenchyma following termination of the study. Initially, studies were carried out in non-tumor-bearing rats to define the relationships among the volume of infusion (V_i), the volume of distribution (V_d), and the rate of infusion. These were divided into four groups of eight animals each as follows: group 1 received an i.c. injection of 5 μCi/50 μg BD-L8A4/10 μL and groups 2 to 4 received BD-L8A4 by CED at a rate of 0.33 μL/min for 15, 30, or 60 minutes with corresponding injection volumes of 5, 10, and 20 μL, respectively. The infusion rates and injection volumes in the brains of non-tumor-bearing rats are summarized in Table 1.

Following completion of these studies, biodistribution studies were carried out in tumor-bearing animals 12 to 14 days following tumor implantation. Animals were divided into four experimental groups of 10 to 16 rats each. Animals in groups 1 and 2 had F98$_{npEGFRvIII}$ gliomas and those in groups 3 and 4 had F98$_{WT}$ tumors. Rats in groups 2 and 4 received an i.t. injection of [^{125}I]BD-L8A4 (5 μCi/50 μg L8A4/10 μL) and those in groups 1 and 3 received an equal volume of [^{125}I]BD-L8A4 delivered by CED over 30 minutes at a rate of 0.33 μL/min. The biodistribution of [^{125}I]BD-L8A4 in tumor-bearing animals was determined at 6, 12, 24, and 48 hours after administration. Animals were euthanized by an overdose of halothane following which tumors and normal tissues, consisting of brain, blood, liver, kidney, and muscle, were removed and weighed. Uptake of ^{125}I was determined by γ-scintillation counting for [^{125}I]BD-L8A4.

Quantitative autoradiographic analysis of distribution of [^{125}I]BD-L8A4 in non-tumor-bearing rats. Rats were euthanized either immediately following or 12 hours after CED and their brains were removed and frozen in isopentane (2-methylbutane), which had been cooled to −150°C in liquid nitrogen, and they were stored at −70°C until sectioning. Brains were cut coronally at 1-mm intervals rostral and caudal to the point of insertion of the cannula. Five serial sections from each coronal slice were cut on a cryostat (Miles Scientific, Naperville, IL) at a thickness of 20 μm each. One of the five sections was stained with H&E for histologic examination. The percent uptake of radioactivity was quantified in two sections by γ-scintillation counting for ^{125}I using a well counter (model 1185, Tm Analytic). The remaining two sections were processed for quantitative autoradiography by exposing them to either NTB-2 dipping emulsion or X-ray stripping film (Eastman Kodak, Rochester, NY). Following a 16-hour exposure, the autoradiographs were developed. ^{125}I standards for quantitative

autoradiography were prepared from homogenized brain at concentrations ranging from 0.01 to 0.8 relative to the ^{125}I concentration of the infusate (12).

The autoradiographs subsequently were analyzed by quantitative densitometry using a Macintosh-based computer image analysis system (Image 1.5, kindly provided by the NIH, Bethesda, MD, via the Internet). The absorbances of the radioactivity standards were fit to known tissue equivalents using a Rodbard function as described previously by us (12). The Wilcoxon rank-sum test in SAS version 8-02 (SAS Institute, Cary NC) was used to calculate exact Ps for tumor uptake of [^{125}I]BD-L8A4. The V_d was defined as the tissue volume whose local concentration of the infused (V_i) [^{125}I]BD-L8A4 relative to the concentration of the infusate uniformly equaled or exceeded an arbitrary fraction (>1%) of the concentration of the infusate. To define the boundaries of infusion, a threshold equal to ~15% of the maximum tissue equivalent was used. The V_d was estimated by multiplying the area of perfusion, as measured by computer analysis, by the distance between sections and summing across all slices. The percent recovery was determined by obtaining the counts/min of ^{125}I in the tissue sample using a Tm Analytic γ-scintillation counter. The total amount of radioactivity in the five coronal sections and the percent recovery were determined as described previously (12). Homogeneity of delivery was determined from autoradiographs made from coronal sections of the brains of animals that had received [^{125}I]BD-L8A4, and cross-sectional concentration profiles were generated. The tissue concentrations of [^{125}I]BD-L8A4, in sequential 0.1-mm increments, were determined by converting the optical densities of the infused regions of the autoradiographs to tissue equivalents (μCi/g tissue) by appropriate ^{125}I standards and image analysis software. Tissue uptake of [^{125}I]BD-L8A4 was determined by γ-counting of weighed samples of blood, liver, kidney, muscle, and skin from each animal.

Therapy experiments and dosimetry. BNCT was done 14 days following stereotactic implantation of 10^3 F98$_{npEGFRvIII}$ glioma cells. Rats were transported to the Nuclear Reactor Laboratory at the Massachusetts Institute of Technology and then randomized based on weight into experimental groups of 7 to 11 animals each as follows: group 1, CED of BD-L8A4 and BNCT; group 2, CED of BD-L8A4 plus i.v. BPA and BNCT; group 3, i.v. BPA and BNCT; group 4, CED of L8A4 and neutron irradiation; and group 5, CED of BD-L8A4. BNCT was initiated 24 hours after CED of 10 μL BD-L8A4 (40 μg ^{10}B/750 μg L8A4) and 2.5 hours after i.v. administration of BPA (500 mg/kg body weight). All irradiated rats were anesthetized with a mixture of ketamine and xylazine. BNCT was carried out at the Massachusetts Institute of Technology Research Reactor-II nuclear reactor in the M011 irradiation facility, which produces a beam of thermal neutrons of high purity and intensity with no measurable fast neutron component. Two rats at a time were positioned in a ^{6}Li-enriched polyethylene box that provided whole-body shielding from the thermal neutrons during irradiation. The animals' heads were aligned in the middle of a 13 × 2 cm^2 aperture, machined in the box lid, which served as the beam delimiter. Four fission counters, located at the periphery of the 15-cm circular field,

Table 1. V_d of [^{125}I] BD-L8A4 following i.c. injection or CED in non-tumor-bearing rats

Group	Route	Infusion			V_d (mm^3)	V_d/V_i ratio	Recovery of infusate
		Time (min)	Volume (mm^3)	Rate (mm^3/min)			
1	i.c.	2	10.0	5.0	10.8 ± 3.1	1.1 ± 0.3.	87.1 ± 6.8
2	CED	15	5.0	0.33	31.6 ± 5.8	6.3 ± 1.2	90.1 ± 8.2
3	CED	30	10.0	0.33	60.8 ± 11.4	6.1 ± 1.3	88.3 ± 5.6
4	CED	60	20.0	0.33	118.5 ± 19.2	5.9 ± 0.6	85.9 ± 4.3

NOTE: V_d was determined by quantitative autoradiography, and percentage recovery was determined by γ-scintillation in groups of four animals each. Means and SDs of V_d, V_d/V_i ratios, and percentage recovery were calculated.

automatically controlled beam delivery and provided real-time data on the relative neutron fluence during an irradiation.

Dosimetric measurements were done using bare gold foils and a graphite-walled ionization chamber ($V = 0.1$ cm^3) flushed with reagent-grade CO_2 on both dead rats and phantoms made from type 6 nylon (36). The measured dose rates in brain (2.2% nitrogen by weight), normalized to the reactor operating at a power of 5 MW, were 18.5 cGy/min for photons, 7.7 cGy/min for thermal neutrons from the nitrogen capture reaction, and 3.4 cGy/min/µg ^{10}B in tissues. For dosimetric calculations, boron concentrations were determined in tumor, normal brain, liver, and blood in a separate group of animals 24 hours after CED of BD-L8A4 and 2.5 hours after i.v. injection of BPA. Animal irradiations were done with the reactor operating at a power between 4.0 and 4.8 MW. These took between 6.9 and 8.6 minutes to deliver a thermal neutron fluence of 2.64×10^{12} n·cm^{-2} to complement previous dose prescriptions (13, 14). After completion of BNCT, the animals were held at Massachusetts Institute of Technology for ~3 days to allow induced radioactivity to decay before they were returned to The Ohio State University for clinical monitoring.

Monitoring of clinical status and neuropathologic evaluation. All animals were weighed three times per week and their clinical status was evaluated at the same time. Once the animals had progressively growing tumors, as evidenced by the combination of sustained weight loss, ataxia, and periorbital hemorrhage, they were euthanized to minimize discomfort. Survival times were determined by adding 1 day to the time between tumor implantation and euthanization. Rats surviving >180 days were designated long-term survivors and were euthanized. The brains of all animals in the therapy studies were removed after death, fixed in 10% buffered formalin, and then cut coronally at the level of the optic chiasma and 2 mm anterior and posterior to it. Tissue sections through the tumor were embedded in paraffin, cut at 4 µm, stained with H&E, and examined microscopically to assess the histopathologic changes. The tumor size index was determined from H&E-stained coronal sections of brain using a semiquantitative grading scale ranging from 0 to 4. Each section was scored as follows: 0, no tumor; 1, very small (i.e., microscopic, <1 mm); 2, small (~1-3 mm); 3, large (~4-7 mm); and 4, massive (>8 mm).

Statistical evaluation of survival data. The mean survival time (MST), SE, and median survival time were calculated for each group using the Kaplan-Meier method (37). The Kaplan-Meier curves and Cox survival curves also were plotted. The hypotheses involved comparing each L8A4 animal to each irradiated control. A log-rank test was used for these comparisons, with a Bonferroni method of adjustment for the multiple comparisons (38). Because five comparisons were tested for statistical significance within the L8A4 tests, $\alpha = 0.0125$ was used.

Results

In vitro *receptor-binding activity of BD-L8A4.* To determine if the immunoreactivity of L8A4 was retained following boronation, BD-L8A4 and unmodified antibody were compared for their binding affinity to F98$_{npEGFRvIII}$ cells. After incubation with either BD-L8A4 or unmodified antibody, F98$_{npEGFRvIII}$ cells were then exposed to [^{125}I]BD-L8A4 for an additional 2 hours. The binding of mAb L8A4 to EGFRvIII was determined by incubating F98$_{EGFRvIII}$ cells, with varying amounts of [^{125}I]L8A4, and data from these experiments are shown in Fig. 1A. [^{125}I]L8A4 had a very low binding affinity with F98$_{WT}$ cells, indicating that nonspecific binding was negligible. In contrast, as determined by binding of [^{125}I]L8A4 to F98$_{npEGFRvIII}$ cells, the affinity constant (K_a) was $4.85 \pm 0.40 \times 10^7$ mol/L^{-1} and receptor site density was $7.34 \pm 0.61 \times 10^5$ sites per cell. These numbers are somewhat higher than the K_a of $6.62 \pm 0.8 \times 10^8$ mol/L^{-1} and a receptor site density of $1.20 \pm 0.08 \times 10^5$ per cell that were reported previously by us (31). As shown in

Fig. 1B, based on the radioactivity bound to F98$_{EGFRvIII}$ cells, it was found that the bioconjugate and unmodified antibody both inhibited binding of [^{125}I]BD-L8A4 in a concentration-dependent manner. The IC$_{50}$ of boronated and native L8A4 were 27.2 and 16.7 nmol/L, respectively, indicating that the bioconjugate had 60% binding activity compared with that of the native antibody.

Intracerebral distribution of [^{125}I]BD-L8A4 in non-tumor-bearing rats. The V_d of [^{125}I]BD-L8A4 following either i.c. injection or CED in normal (i.e., non-tumor-bearing) rat brains are summarized in Table 1. The V_d that contained >1% of the amount of the bioconjugate that was infused were 31.6, 60.8, and 118.5 µL, respectively, following CED of 5, 10, and 20 µL of bioconjugate. The calculated V_d, following i.c. injection of 10 µL, was 10.8 µL and the V_d/V_i ratio was 1.1 compared with 6.3, 6.1, and 5.9 for CED. CED resulted in ~6-fold
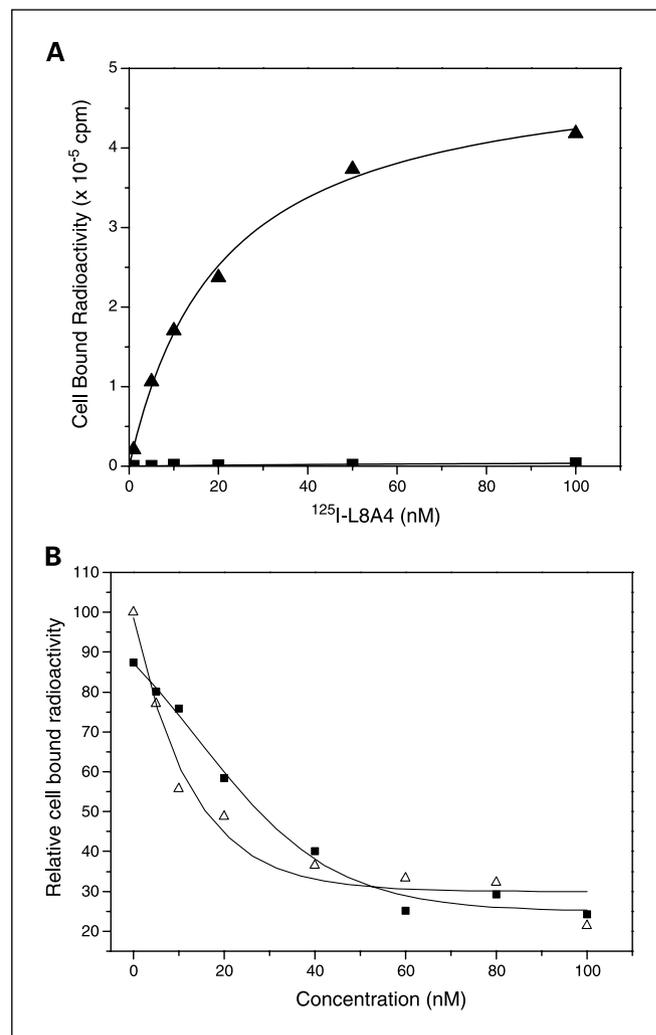
Fig. 1. *A,* cellular binding of mAb L8A4 to EGFRvIII-positive (F98$_{npEGFRvIII}$) and EGFRvIII-negative (F98$_{WT}$) expressing glioma cells. Varying amounts of [^{125}I]L8A4 ranging from 0 to 100 nmol/L were incubated at 4°C for 90 minutes with cells expressing mutant EGFRvIII receptors (F98$_{npEGFRvIII}$; ▲) and receptor-negative parental cells (F98$_{WT}$; ■). Cell-bound radioactivity was determined by γ-scintillation counting. *B,* competitive binding assay using ^{125}I-labeled bioconjugate. Cells were incubated at ambient temperature for 2 hours with medium containing 0.185 nmol/L [^{125}I]BD-L8A4 and varying concentrations (0-500 nM) of either native L8A4 (△) or BD-L8A4 (■) following which cell-associated radioactivity was determined by γ-scintillation counting. Each point represents the mean of six replicates.
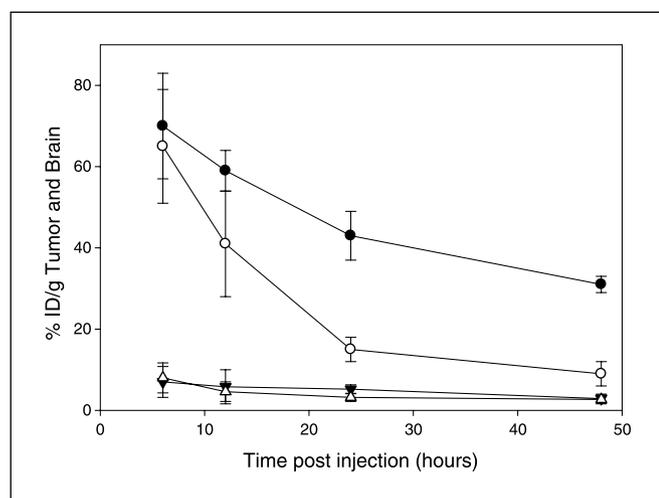
Fig. 2. Tumor and normal brain uptake of ^{125}I-labeled bioconjugate. F98$_{npEGFRvIII}$ (●) or F98$_{WT}$ (○) glioma-bearing rats were injected i.t. with [125I]BD-LA84 and euthanized 6, 12, 24, and 48 hours later. Normal brain uptake in F98$_{npEGFRvIII}$ (▼) and F98$_{WT}$ (△) tumor-bearing rats was determined following excision of the tumor. *Points,* mean of four rats; *bars,* SD.

increase in the V_d/V_i ratio compared with that attained following i.c. injection. These differences were clearly evident in a series of autoradiographs (data not shown) that were made from coronal sections of brains from non-tumor-bearing rats. As determined by quantitative densitometry, immediately after completion of CED of 20 μL [125I]BD-L8A4 for 60 minutes, ~80% of the rat gray matter or ~25% of the infused hemisphere had received >1% of the infusion concentration. The mean percent radioactivity recovered from the brain were 90.1%, 88.3%, and 85.9%, respectively, for V_i of 5, 10, and 20 μL (Table 1). The V_d of animals euthanized 12 hours after CED or i.c. injection were slightly increased over those determined immediately after CED or i.c. injection (data not shown). The concentration of the bioconjugate in normal brain, as determined by quantitative autoradiography, was homogenous. Microscopic examination of the brains of non-tumor-bearing rats, which had undergone CED, showed minimal hemorrhage along the path of the infusion cannula but no other neuropathologic changes in the remainder of the infused cerebral hemisphere.

Biodistribution of [125I]BD-L8A4 in glioma-bearing rats. Tumor and normal tissue uptake of the bioconjugate following either i.t. injection or CED into glioma-bearing rats is summarized in Fig. 2 and Table 2. As determined by γ-scintillation counting, between 1 and 6 hours following i.t. injection or CED, 60% to 80% ID/g of BD-L8A4 was nonspecifically localized in F98$_{npEGFRvIII}$ gliomas and the differences between the two groups were not statistically significant (Fig. 2). However, by 12 hours following i.t. injection, retention of BD-L8A4 in F98$_{npEGFRvIII}$ gliomas was higher than that of F98$_{WT}$ tumors (58.5% versus 41.3% ID/g) and these differences became larger at 24 hours (43.7% versus 15.2% ID/g) and 48 hours (31.3% versus 8.5% ID/g; $P < 0.01$). In contrast, there were no significant differences in the amount of the bioconjugate retained in normal brain (Fig. 2). Similar differences were noted in animals that received BD-L8A4 by CED. At 24 hours following CED, 60.1% ID/g was localized in F98$_{npEGFRvIII}$ gliomas compared with 43.7% ID/g after i.t. injection and 14.6% ID/g after CED in F98$_{WT}$ gliomas (Table 2). Using the Wilcoxon rank-sum test to calculate exact P values, the differences between the CED and i.t. groups were highly significant ($P = 0.008$) as were differences between the F98$_{npEGFRvIII}$ and F98$_{WT}$ groups. The amounts of radioactivity in muscle, liver, skin, and kidney after CED were all <1% of the injected dose for all infusion volumes and times after infusion. Autoradiographs clearly showed that CED was far superior to i.t. injection as a means to disperse [125I]BD-L8A4 within the tumor. Although the brains of glioma-bearing rats showed significant tumor-related neuropathologic changes, none seemed to be related specifically to insertion of the cannula. Tumor weights ranged from 160 to 300 mg, and as determined from H&E-stained coronal sections, tumor diameters ranged from ~3 to 7 mm.

Tissue boron concentrations and dosimetry. Boron concentrations in tumor and selected normal tissues and the calculated physical doses delivered to them are summarized in Table 3. The tumor boron concentration in rats that received CED of BD-L8A4 was 32.7 ± 3.6 μg/g compared with 10.7 ± 1.7 μg/g following i.v. injection of BPA. Boron levels in normal tissues were in the undetectable range (<0.5 μg/g) in rats that received CED of BD-L8A4. Dosimetric calculations were based on mean boron concentrations of tumor, brain, and blood at 24 hours following CED of BD-L8A4 and 2.5 hours after i.v. administration of BPA in a separate group of untreated animals. Based on these total boron concentrations, the mean absorbed dose delivered to F98$_{npEGFRvIII}$ tumors was 9.2 Gy following CED of L8A4, 4.2 Gy following i.v. administration of BPA alone, and

Table 2. Radiolocalization of [125I]BD-L8A4 in F98 glioma-bearing rats at 24 hours following either i.t. injection or CED

Tumor/route	%ID/g					Tumor/brain ratio
	Tumor	**Brain ipsilateral**	**Brain contralateral**	**Liver**	**Blood**	
F98$_{npEGFRvIII}$/CED	60.1 ± 10.8	6.2 ± 1.2	2.0 ± 0.5	0.19 ± 0.05	0.05 ± 0.01	9.5 ± 2.6
F98$_{npEGFRvIII}$/i.t.	43.7 ± 6.1	5.6 ± 1.1	2.9 ± 1.1	0.19 ± 0.05	0.06 ± 0.03	6.9 ± 1.3
F98$_{WT}$/CED	14.6 ± 2.7	2.9 ± 1.2	1.2 ± 0.5	0.22 ± 0.08	0.05 ± 0.01	5.6 ± 2.6
F98$_{WT}$/i.t.	15.2 ± 3.4	3.2 ± 1.0	1.0 ± 0.5	0.34 ± 0.06	0.10 ± 0.02	4.9 ± 1.0

NOTE: Each animal received 5 μCi [125I]BD-L8A4 (50 μg L8A4) by either i.t. or CED. %ID/g was based on the amount recovered relative to that given. The tumor/brain ratio was based on tumor uptake versus ipsilateral (tumor-bearing) cerebral hemisphere.

Table 3. Boron concentrations and radiation absorbed dose to tumor, brain, and blood

Group	Boron concentrations (μg/g)*			Absorbed dose (Gy)[†]		
	Tumor	Brain[‡]	Blood	Tumor	Brain	Blood
CED BD-L8A4	32.7 ± 3.6	<0.5	<0.5	9.2	1.9	1.9
i.v. BPA	10.7 ± 1.7	3.8 ± 1.1	5.2 ± 1.3	4.2	2.6	2.9
CED BD-L8A4 i.v. BPA	44.5 ± 11.1	4.2 ± 0.5	4.0 ± 1.2	12.0	2.7	2.7

*Boron content was quantified by means of direct current plasma-atomic emission spectroscopy. These values were obtained from rats that had received CED of BD-L8A4 (40 μg ^{10}B/750 mg L8A4) alone 24 hours earlier or in combination with i.v. BPA (500 mg/kg body weight equivalent to 27 mg ^{10}B/kg body weight), which was given 2.5 hours before euthanization.

[†] Absorbed dose determinations include contributions from γ-photons, ^{14}N(n,p)^{14}C and ^{10}B(n,α)^{7}Li reactions.

[‡] Boron concentrations were determined in the tumor-bearing cerebral hemisphere after excision of the tumor.

12.0 Gy in combination with CED of BD-L8A4 (Table 3). The normal brain doses ranged from 1.9 to 2.7 Gy. All doses in this report were expressed as the physical absorbed dose and no attempt was made to apply biological weighting factors.

Therapeutic response of glioma-bearing rats following BNCT. All animals in a pilot study to determine tolerance to BNCT following CED of BD-L8A4 lost weight within 7 to 10 days after treatment. Rats that received CED of ~40 μg ^{10}B/ ~750 μg BD-L8A4 and 500 mg/kg body weight of BPA i.v. lost <10% of their body weight but regained it within 2 weeks. Based on these results, CED of ~40 μg ^{10}B/~750 μg L8A4 alone or in combination with i.v. BPA was used. BNCT was initiated at the Massachusetts Institute of Technology Research Reactor-II 14 days following i.c. implantation of 10^3 F98$_{npEGFR-vIII}$ glioma cells. All rats tolerated BNCT without any untoward effects, and 3 to 7 days later, they were returned to Columbus, OH. A Cox proportional hazards regression model was fit to the data and the proportional hazards assumption was checked. Because this was met, the log-rank test was used to test for significance between survival curves, and the survival data of the groups were significantly different overall ($P < 0.0001$), indicating that not all of the Kaplan-Meier survival plots were equal. Survival data following BNCT are summarized in Table 4 and Kaplan-Meier and Cox survival plots for BNCT-treated animals and irradiated controls are shown in Figs. 3 and 4, respectively. Untreated control rats had a MST ± SE of 26.3 ± 1.6 days compared with a modest increase of 30.3 ± 1.6 days

for the irradiated controls (Table 4). Animals bearing F98$_{npEGFRvIII}$ gliomas, which had received CED of BD-L8A4 and BNCT, had a MST of 70.4 ± 11.1 days (range, 41->180 days), with one animal surviving 180 days. Animals that received CED of BD-L8A4 in combination with i.v. BPA had a MST of 85.5 ± 15.5 days (range, 42 to >180 days) with two rats surviving >180 days compared with 40.1 ± 2.2 days (range, 32-52 days) for animals that received i.v. BPA alone. The corresponding percent increased life span were 225.1% for the combination versus 168% for CED of BD-L8A4 and 54% for i.v. BPA (Table 4). The results from these comparisons indicate that CED of BD-L8A4 either alone or in combination with i.v. BPA was significantly different from the i.v. BPA group ($P = 0.02$) and the untreated and irradiated control groups ($P = 0.002$). However, the difference in MSTs between the groups that received BD-L8A4 either alone or combination with BPA was not significant ($P = 0.15$). This lack of statistical significance was attributable to the overlapping ranges in survival times of animals that received CED of BD-L8A4 alone (41-180 days) versus those that received it in combination with i.v. BPA (42-180 days).

Neuropathologic findings. The brains of all animals were subjected to histopathologic examination. Animals that received boronated L8A4 either alone or in combination with BPA had almost identical tumor size indices (3.6, 3.8, and 3.8). Control animals that received either non-BD, which had been conjugated to L8A4, or BD-L8A4 without neutron irradiation

Table 4. Survival times of F98$_{npEGFRvIII}$ glioma-bearing rats following CED or i.t. injection of BD-L8A4 with or without i.v. BPA and BNCT

Group	Route/agent	Survival times (d)			% Increased life span*	
		Range[†]	Mean ± SE	Median	Mean	Median
1	CED/BD-L8A4 (*n* = 11)	41 to >180 (*n* = 1)	70.4 ± 11.1	58	168	123
2	CED/BD-L8A4 + i.v./BPA (*n* = 10)	42 to >180 (*n* = 2)	85.5 ± 15.5	65.5	225	152
3	i.v./BPA (*n* = 8)	32-52	40.1 ± 2.2	40	52	54
4	CED/L8A4 + irradiation (*n* = 7)	24-37	30.3 ± 1.6	30	15	15
5	CED/BD-L8A4 (*n* = 7)	21-34	26.3 ± 1.6	26	—	—

*Percentage of increased life span was defined relative to MST and median survival time of untreated controls.

[†] *n* is the number of rats surviving >180 days.
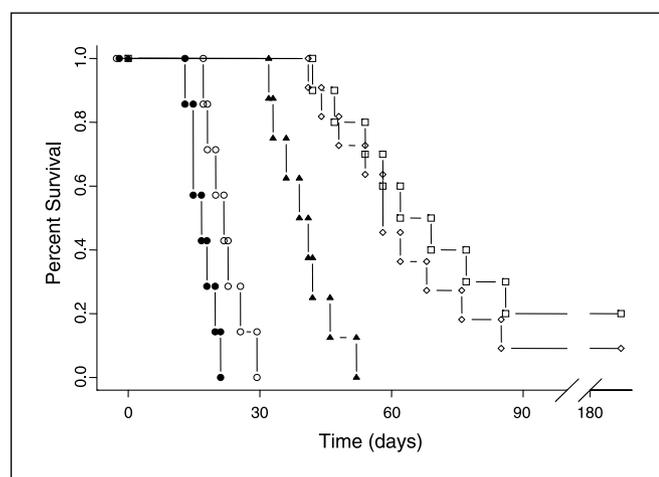
Fig. 3. Kaplan-Meier survival plots for F98$_{npEGFRvIII}$ glioma-bearing rats. Survival times in days after implantation have been plotted for untreated animals (●), irradiation + L8A4 (○), i.v. BPA + BNCT (▲), and CED of BD-L8A4 alone (◇) or in combination with i.v. BPA (□) followed by BNCT. The differences in MSTs of those animals that received BD-L8A4 either alone or in combination with BPA were significantly different ($P < 0.01$) from those of untreated or irradiated control animals or BPA alone ($P < 0.02$). However, they were not different from each other ($P = 0.16$).

had smaller tumor size indices (3.4 and 3.0, respectively). The fact that these animals died with smaller tumors than those that had received BNCT suggests that they may have had more cerebral edema, which invariably is associated with brain tumors (39).

Although there was considerable variability in the histopathologic appearance of the tumors at the time of the animal's death or euthanization, there were certain constant features irrespective of the treatment that they had received (Fig. 5A and B). Almost all of the tumors were highly infiltrative of surrounding white matter with islands of malignant cells at some distance from the main tumor mass. There were only small zones of necrosis either located centrally or in the periphery. There was variability in cellular morphology, ranging from spindle-shaped cells showing a storiform pattern of growth to contiguous sheets or clusters of small, round cells showing a highly infiltrative pattern of growth (Fig. 5B), which is characteristic of malignant gliomas (40). In addition, rare tumor giant cells and occasional mitotic figures were seen (Fig. 5B). This cellular pleomorphism resembled that seen in human high-grade gliomas (40). Immunostaining revealed that the tumor cells were glial fibrillary acidic protein negative and S100 negative. Rare CD3$^+$ T lymphocytes were seen within the tumor and scattered CD68$^+$ macrophages were seen in the periphery. In contrast to these findings, the brain of one long-term survivor (i.e., >180 days), which had received BD-L8A4 plus BPA, showed no evidence of residual tumor, necrosis, or inflammatory cell infiltrates. The brains of two other long-term survivors showed focal fibrosis, with small (<1 mm) central cavities (Fig. 5C and E). There were CD3$^+$, CD20$^+$, CD68$^+$, and glial fibrillary acidic protein-positive cells and hemosiderin laden macrophages interspersed in the fibrous tissue component (Fig. 5D and E). In the brain of one of these animals, there was a dense infiltrate of polymorphonuclear leukocytes adjacent to the fibrous tissue (Fig. 5F). Because a tumor-associated immune response would have a lymphocytic infiltrate,

these most likely were the result of an acute inflammatory response that may have been evoked by bacteria that were inadvertently introduced into the brain at the time of tumor cell implantation due to bacterial contamination of the syringe needle. Choroidal epithelial cells, which are especially sensitive to radiation injury, appeared to be normal. In summary, these histopathologic changes were similar to those that we have seen in long-term survivors from other studies (41) and showed that tumor cells could be selectively killed without damage to adjacent normal brain.

Discussion

In the present study, we have evaluated the efficacy of the anti-EGFRvIII mAb L8A4 as a boron delivery agent for NCT in rats bearing i.c. implants of the F98$_{npEGFRvIII}$ glioma. We first established that CED more efficiently delivered BD-L8A4 to the tumor compared with direct i.t. injection (60.1% versus 43.7% ID/g). Based on these observations, CED was used to administer the bioconjugate in our therapy experiments. Boronated L8A4 was given either alone or in combination with i.v. injection of BPA, and 24 hours later, BNCT was carried out. The MST of animals that received the bioconjugate in combination with BPA was 85.5 days compared with 70.4 days for those that received it alone, 40.1 days for BPA alone, and 30.3 days for irradiated controls. Among the treated animals, there was a subset of rats that survived >180 days, which clinically were regarded as cured, and this was confirmed by histopathologic examination. These survival data were superior to those that we have obtained using BPA in combination with BSH following i.v. or intra-arterial administration (41, 42). The present data, and those recently reported by us in a preliminary study using boronated cetuximab (14), establish proof-of-principle that gliomas expressing either EGFR or EGFRvIII can be selectively targeted with boronated mAbs, given by either i.t. injection or CED, and that a significant gain in survival time can be obtained following NCT.

To date, only two drugs have been used in clinical BNCT trials, BSH and BPA (1). The potential use of high molecular
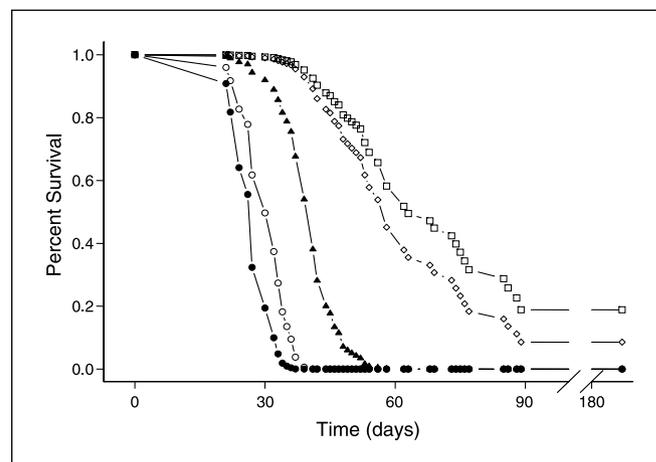
Fig. 4. Cox survival plots for F98$_{npEGFRvIII}$ glioma-bearing rats. Survival times in days after implantation have been plotted for untreated animals (●), irradiation + L8A4 (○), i.v. BPA + BNCT (▲), and CED of BD-L8A4 alone (◇) or in combination with i.v. BPA (□) followed by BNCT.
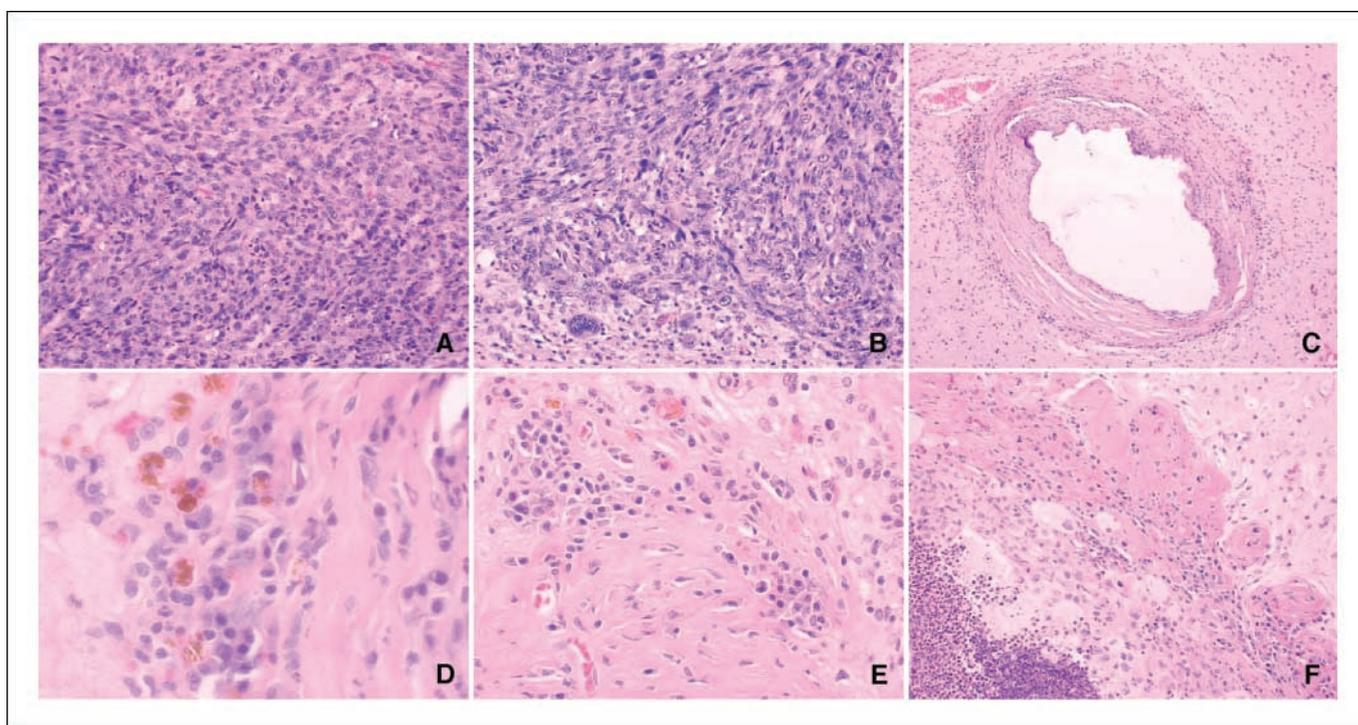
Fig. 5. Neuropathologic findings in untreated and BNCT-treated rats bearing i.c. implants of the F98$_{npEGFRvIII}$ glioma. *A,* tumor in an untreated rat. This was composed predominantly of spindle-shaped cells showing a storiform pattern of growth. Magnification, ×200. *B,* tumor from a rat that had received i.v. BPA. There was considerable cellular pleiomorphism ranging from spindle-shaped cells in the main tumor mass to small round cells and occasional multinucleate tumor giant cells in the periphery. Magnification, ×200. *C,* brain of a long-term survivor (180 days) showing a small central cavity (<1 mm) surrounded by fibrotic tissue (magnification, ×100), in which (*D*) there were lymphocytes and macrophages, some of which were hemosiderin laden but no residual tumor cells (magnification, ×600). *E,* the brain of another long-term survivor showed a circumscribed area of fibrosis with scattered macrophages and rare lymphocytes. Magnification, ×400. *F,* in another area of this same tumor, there was a focal infiltrate of polymorphonuclear leukocytes. Magnification, ×200.

weight boron delivery agents recently has been reviewed by us (43), but none of these have as yet been evaluated clinically. Studies previously carried out by us have shown that the i.v. route of administration was unsuitable for the delivery of boronated EGF (10, 19) and L8A4 (31) in glioma-bearing rats. One way to overcome the blood-brain barrier is to administer the high molecular weight agents directly into the brain by CED (44–46). This approach has been used by us in the present study, and as recently reported, it has been used clinically to administer the anti-tenascin mAb 81C6 into the cavity created following surgical resection of gliomas (47). The efficacy and toxicity of [^{131}I]81C6 was evaluated in a group of 43 patients with recurrent high-grade gliomas. The mAb was given by Rickham reservoirs and cannulas that had been placed into the resection cavity. The median overall survival of patients with glioblastoma multiforme and anaplastic astrocytomas were 64 and 99 weeks, respectively, which were greater than those of historical controls at the same institution (Duke University Medical Center, Durham, NC) for patients that had been treated with surgery plus ^{125}I brachytherapy. These studies have established the feasibility of using direct i.c. delivery of a mAb directed against a tumor-associated antigen, and based on these favorable results, a phase III multicenter trial is planned (47). Our own data suggest that the use of boronated antibodies is feasible for NCT. A significant advantage of a boronated delivery agent over the direct administration of a radiolabeled mAb is that one can wait until the ^{10}B has cleared from normal tissues before BNCT is carried out, thereby eliminating or minimizing damage to normal brain.

The F98$_{npEGFRvIII}$ glioma, used in this study, expressed human EGFRvIII at a density of >10^5 receptor sites per cell. Another cell line, designated F98$_{EGFRvIII}$, which has been produced for us by Drs. Frank Furnari and Webster Cavenee (Ludwig Institute for Cancer Research, University of California at San Diego, San Diego, CA), expressed >10^6 receptor sites per cell. Furthermore, this receptor was constitutively phosphorylated compared with the nonphosphorylated receptor of the F98$_{npEGFRvIII}$ cell line that we have used in the present study. However, the former cell line, when implanted i.c. with logarithmically incremental numbers of tumor cells, did not produce survival times that were a linear function of the tumor cell dose, and unexpectedly, animals that received 10^5 tumor cells i.c. had longer survival times than those that received 10^3 cells.[9] Based on these observations, and our previous studies with another EGFR transfectant model, the C6 glioma, which had been transfected with the human EGFR gene (48), we have concluded that the cell line expressing 10^6 EGFRvIII sites per cell can evoke an immune response directed against the human receptor, whereas the F98$_{npEGFRvIII}$ cell line does not. Although it would be advantageous to have a cell line that had a functional (i.e., phosphorylated) receptor expressing 10^6 receptor sites per cell, it would not be possible to use the one that Furnari and Cavenee have produced for experimental therapy studies in syngeneic Fischer rats because the ensuing immune response would confound the effects of

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[9] W. Yang and R.F. Barth, unpublished data.

the therapeutic agent. To prevent this, it would be necessary to use immunologically deficient nude rats to obviate the immune response that would occur in immunocompetent animals.

We would like to make several comments relating to the neuropathologic findings in those rats that clinically were cured and relate them to BNCT. First, it is noteworthy that there was no evidence of normal brain necrosis or small blood vessel injury at the site of infusion of the boronated L8A4. This indicated that the ^{10}B levels in normal brain at 24 hours following CED had fallen below the threshold amounts that would have been required to produce radiation injury (49). Second, the very fact that we had a subset of animals that were histopathologically free of tumor strongly suggests that all or almost all of the tumor cells had been killed by the α-particles and recoiling ^{7}Li particles that were produced in the ^{10}B(n,α)^{7}Li capture reaction. Bystander effects following α-particle irradiation have been described by several investigators (50–53) and we believe that our data show that following BNCT molecular targeting of a tumor cell surface receptor with a boronated mAb was tumoricidal either directly or via bystander effects. The challenge is to develop other molecular targeting agents that can selectively deliver ^{10}B to tumor cells.

The data that we have obtained with the mAbs L8A4 and cetuximab define two characteristics of EGFR targeting bioconjugates that make them promising boron delivery agents for use in combination with either BPA alone or with BPA and BSH as is currently being evaluated in Japan (54). The data shown in Tables 3 and 4 indicate that CED of BD-L8A4 was more effective than i.v. administration of BPA for BNCT of the F98$_{npEGFRvIII}$ glioma. When the MSTs of animals in the experimental and control groups were plotted versus the ^{10}B concentrations in the tumor, there was a linear relationship (r^2 = 0.998). This indicated that the MST was directly related to the total cell-associated boron in the tumor and that the radiobiological effectiveness of the ^{10}B contributed by the bioconjugate was additive to that of BPA. However, use of the boronated conjugate allowed us to escalate the tumor ^{10}B concentration without significantly increasing the blood and normal brain concentrations. In contrast, when combinations of BPA and BSH were used, there always was an increased amount of ^{10}B in the blood and normal tissues (41, 42), which limited the neutron dose that could be delivered to the target volume. BPA results in tumor-to-normal brain boron concentration ratios that may be as high as 3:1 or 4:1, but the boron concentration in normal brain is still significant, which limits the neutron dose that can be delivered to the tumor (2). Because BSH does not cross the intact blood-brain barrier, it may contribute additional ^{10}B to the tumor, but the tumor-to-blood boron concentrations ratios with BSH are ~1:1, thereby limiting the neutron dose in clinical irradiations (2).

We now have evaluated three boronated EGFR targeting agents, the ligand itself, EGF (11, 13), cetuximab (14), and, as reported in the present study, L8A4. Based on all of our data, what can we conclude about these three different targeting agents? EGF bioconjugates only can target the wild-type receptor, and the L8A4 bioconjugate only can target EGFRvIII. Cetuximab, on the other hand, can target both wild-type EGFR and EGFRvIII, although its binding affinity for the latter was less than that of L8A4.[10] What are the advantages of each? EGF bioconjugates have better diffusion properties within the tumor by virtue of their lower molecular weight. L8A4 bioconjugates have specificity only for EGFRvIII and not wild-type EGFR, which is expressed on many different types of cells, and this could be of importance if it were given systemically. However, only cetuximab (Erbitux) has met rigorous Food and Drug Administration requirements for biologics and is now being widely used clinically (55–58), whereas L8A4 and EGF only have been evaluated in experimental animals studies, have not been prepared in good manufacturing practice (GMP) facilities, and are very costly to produce. The conclusion at this time, therefore, is that boronated cetuximab (14, 15), which will be the subject of another report, would be the agent of choice.

We would like to conclude with some comments relating to the clinical trials that have been carried out with BSH (59) and BPA (60–63) as single agents, the results of which have been reported and discussed in several recent reviews and monographs (1–3). In the clinical trials carried out with BPA at the Brookhaven National Laboratory in the United States (60), there were not only regions of frank tumor necrosis but also evidence of tumor progression in the high-dose target volume, suggesting that there was nonuniform distribution of boron to the tumor cells. Longer infusions of BPA, carried out in Sweden (63), may marginally have increased the median survival time, but the dose escalation studies with BPA have reached the point where normal brain tolerance has become the limiting factor. The occurrence of the somnolence syndrome in patients treated with the higher radiation doses has been reported (64), which indicates that dose escalation trials with BPA as the single boron delivery agent, have reached a limit both in terms of the doses of BPA and neutrons. With BPA, there was a significant amount of boron in normal brain, and this undoubtedly contributed to the brain dose and the occurrence of the somnolence syndrome. Further progress in clinical studies of BNCT for high-grade gliomas will require significant improvements in the methods used to administer BPA and BSH, as well as the use of combinations of agents, which is based on the premise that two or more boron delivery agents would target different subpopulations of tumor cells. We have shown that the combination of BPA and BSH was more effective than either one alone in BNCT of the rat F98 glioma (41, 42). Ono et al. have reported similar data for BNCT of the SCCVII rat squamous cell carcinoma (65). However, although BPA and BSH have been shown to be clinically safe, their therapeutic efficacy, at least as they are currently being used, is less than ideal (66). The time has come to move on to new boron delivery agents (67), among which high molecular weight targeting bioconjugates (68) may be particularly promising candidates. Because it is highly unlikely that all tumor cells within a glioblastoma multiforme would express either EGFR or EGFRvIII, the bioconjugate would have to be used in combination with BPA alone or with BSH.

Acknowledgments

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[10] G. Wu and R.F. Barth, unpublished data.

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Clin Cancer Res 2006;12:3792-3802.

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