Intracerebral transplantation of a human glioma line in immunosuppressed rats

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A model was developed for in vivo study of the human glioma-derived D-54 MG cell line in the brains of immunosuppressed Fischer 344 rats. The rats were injected with horse anti-rat thymocyte serum before and after intracerebral inoculation with 5 or 10 μl of a D-54 MG tumor cell suspension. Reproducible mortality distributions were obtained, with deaths occurring 18 to 34 days after intracerebral inoculation. Tumors grew as well circumscribed intracerebral masses with sheets of anaplastic cells, areas of necrosis bordered by concentrated nuclei, and minimal lymphocytic infiltration. Cytogenetic analysis revealed the same general chromosome distribution and markers in the heterotransplanted glioma cells as in the cultured line. Blood-brain barrier disruption was demonstrated by intracerebral tumor staining after intravenous injection of Evans blue dye. The in vivo growth of D-54 MG in immunosuppressed rats provides a reliable experimental model for the study of chemotherapy, immunodiagnosis, and immunotherapy of a human glioma-derived tumor in an animal sufficiently large to evaluate intracarotid or intratumoral injection of therapeutic agents.

KEY WORDS • glioma transplant • experimental tumor • immunosuppression • chromosome markers • brain-tumor model • tissue culture

ALTHOUGH modest gains have been made in the treatment of malignant glial tumors, the prognosis continues to be poor, with a median life expectancy of less than 1 year after diagnosis.12,26 New forms of therapy are needed. In most cases new therapy should be evaluated in animal glioma models prior to use in Phase I clinical trials. In the past, these animal gliomas have been induced with viruses or chemicals, or transplanted from homologous or heterologous donors.22 However, the scope and applicability of experiments with such models may be limited due to the non-human character of the tumors or the small size of athymic mice in which human gliomas have been successfully transplanted. We report the development from the human glioma-derived cell culture line D-54 MG10 of a xenotransplantable glioma line in the brain of immunosuppressed rats. The model will be used for future investigation in brain-tumor diagnosis and treatment.

Materials and Methods

Tumor Cell Suspensions and Media Preparation
A human glioma-derived permanent cell line, D-54 MG, was used in all experiments. It had been passed 73 times in tissue culture before transplantation into nude Sprague-Dawley mice (nu/nu genotype, BALB/c background).* Tumors used for experiments were obtained from nude mouse passage levels 13 to 18. The mice were maintained as described previously.6 Fourteen to 21 days after subcutaneous inoculation, the mice were killed by cervical dislocation, and their tumors removed under sterile conditions. Tumor fragments were minced with dissecting scissors, and passed through a modified tissue press with a bilayered 40/60 mesh cytosieve. This was mixed with Richter's improved zinc option minimal essential medium, passed through an 80-mesh cytosieve, and spun at 250 G for 5 minutes. Supernatant was decanted, and the remaining portion was combined in a 1:1 volume ratio with 0.1% methyl cellulose dissolved in media without serum.

The production of the anti-rat thymocyte serum (ATS) has been described by Denlinger, et al.7 Normal horse serum (NHS)† was given as a control injection to animals not receiving ATS.

* Sprague-Dawley mice obtained from ARC Sprague-Dawley, Madison, Wisconsin.
† Normal horse serum obtained from Gibco Laboratories, Grand Island, New York.
Intracerebral glioma transplant in immunosuppressed rats

Radiation

Radiation was generated by a 250-kV Maximar III therapy unit‡ at an exposure rate of 50 rads/min. In a preliminary experiment, 70 Fischer CDF rats, each weighing 130 to 160 gm, were divided into six groups, and one group each received 550, 600, 650, 700, 750, or 800 rads exposure. Four (27%) of the 15 rats in the 800-rad group died 2 to 5 weeks after radiation; no deaths occurred in the 550- to 750-rad groups. Another group of 120- to 140-gm Fischer rats was subsequently given 750 rads and ATS prior to intracerebral inoculation of D-54 MG tumor. Mortality greater than 50% due to progressive cachexia rather than tumor growth was observed within 3 weeks. We reevaluated individual growth curves in our preliminary experiment, and chose 600 rads as a sublethal radiation dose which could be safely used with ATS for immunosuppression prior to tumor transplantation.

Experimental Design

Model 1. The Model 1 protocol involved the immunosuppression of adult rats with radiation and/or ATS. Adult male Fischer CDF 344 rats§ weighing 100 to 160 gm were used. All rats were kept either in isolation rooms or in sterilized containers with polyester bacterial air filters. They were divided into four groups of 10 to 15 animals each. Group A received radiation, ATS, and injection of D-54 MG tumor; Group B received radiation, NHS, and D-54 MG tumor injection; Group C received ATS and D-54 MG tumor injection; and Group D received NHS and D-54 MG tumor injection. This experiment was done twice (Groups A₁-D₁, and A₂-D₂). On Day 1, rats in Groups A and B received 600 rads of whole-body radiation. Later on Day 1, rats in Groups A and C received 50 µl of ATS delivered intraperitoneally. Rats in Groups B and D received 50 µl of NHS. On Day 2, all animals were anesthetized with methoxyflurane gas. A 2-cm incision was made over the sagittal suture, and a hole was placed in the skull with a No. 19 needle 2 mm posterior to the right coronal suture and 2 mm lateral to the sagittal suture. Modified No. 25 needles with 5 mm of exposed cannula were used to inject 10 µl of cell suspension into the area of the right caudate nucleus. All the animals were maintained on ATS or NHS, two injections per week. All animals in Groups A to D that did not die spontaneously were killed on Days 33 or 35. A general necropsy was performed; the heads were removed, stripped of soft tissue, and placed in 10% buffered formalin.

Model 2. The Model 2 protocol called for the immunosuppression of newborn rats with ATS. Ninety 2-day-old Fischer CDF 344 rats were divided into six groups of 10 to 20 animals each. Group A received ATS and a 5-µl D-54 MG tumor injection on Day 7; Group B received NHS and a 5-µl D-54 MG tumor injection on Day 7; Group C received ATS and a 10-µl D-54 MG tumor injection on Day 7; Group D received NHS and a 10-µl D-54 MG tumor injection on Day 7; Group E received ATS and a 10-µl D-54 MG tumor injection on Day 20; and Group F received NHS and a 10-µl D-54 MG tumor injection on Day 20.

On Day 1, all Group A, C, and E rats received 50 µl of ATS. Thereafter, all animals received ATS or NHS twice per week. Due to cannibalism or foster-mother neglect, 21 animals died in the 1st week, leaving 14 animals in Group A, 10 in Group B, 15 in Group C, seven in Group D, 13 in Group E, and 10 in Group F. On Day 7 or 20, animals received an intracerebral inoculation of D-54 MG cell suspension. Survivors in all groups were killed 6 to 7 weeks after tumor injection. At death, the heads were removed and were processed as described for Model 1 animals.

Cytogenic Analysis

Intracranial tumors were dissected free of surrounding brain tissue, finely minced, and enzymatically dissociated using Hanks balanced salt solution (pH 7.0) containing 0.02% collagenase (125 units/mg), 0.05% pronase (45 PKU/mg B grade), and 0.02% DNAase (7 × 10^4 dissociation units/mg of DNAase I B grade) at 37°C for approximately 45 minutes. The cell suspension from each tumor was centrifuged; the pellet was resuspended in 20% fetal calf serum-zinc option medium and plated in four 100-mm dishes. Giemsa-trypsin banding and karyotype analysis were performed as previously described for the D-54 MG tumor in culture.1 Chromosome counts were performed on 50 metaphases per tumor. G-banded chromosomes were arranged according to the International System for Human Cytogenetics Nomenclature.13

Morphological Examination

Rat heads were decalcified in RDO (rapid bond decalculator) for 8 to 12 hours and cut coronally. One to three sections per animal were embedded in paraffin, and 6-µ sections were stained with hematoxylin and eosin. The brains were coded and evaluated for 1) the presence of tumor; 2) tumor size (gross versus microscopic); 3) tumor cell morphology; and 4) the presence or absence of inflammatory infiltrate.

Results

Model 1

In the first experiment with adult Fischer rats, six (86%) of seven Group A₁ animals died of large intra-
cerebral tumors 13 to 19 days after D-54 MG tumor injection (Table 1). Three (33%) of nine Group B1 animals died 7 to 15 days after tumor injection; two had microscopic tumors and one had a large tumor. In the second experiment, 12 (86%) of 14 Group A2 animals died of large intracerebral tumors after 16 to 27 days. One Group B2 animal died after 7 days; it had a microscopic tumor. There were no Group C or D spontaneous deaths in either experiment (Fig. 1). At necropsy of long-term survivors, 33 and 35 days after tumor injection, no animal had intracerebral tumor.

Model 2

Thirty-seven (95%) of 39 neonatal rats immunosuppressed with ATS (Groups A, C, and E) died of large intracranial tumors (Table 2). All 11 (100%) Group A animals, 13 (87%) of 15 Group C animals, and all 13 (100%) Group E animals died 23 to 34, 18 to 30, and 18 to 28 days, respectively, after intracerebral D-54 MG tumor injection. There were no deaths in Groups B, D, or F (Fig. 2). At necropsy of long-term survivors, 6 to 7 weeks after tumor injection, there were no intracerebral tumors present. One moribund rat in Group C was killed 45 minutes after intravenous injection with 0.5 ml of 0.1% Evans blue dye. Examination of its intracerebral tumor revealed marked staining which was absent in surrounding normal brain.

### TABLE 1

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>RT</th>
<th>ATS</th>
<th>NHS</th>
<th>No. of Rats</th>
<th>Spontaneous Deaths</th>
<th>Size of IC Tumor</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Surviving†</td>
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<td>10</td>
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<td>6</td>
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<tr>
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<td>−</td>
<td>+</td>
<td>10</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>C1</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>10</td>
<td>9</td>
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</tr>
<tr>
<td>D1</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>10</td>
<td>10</td>
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</tr>
<tr>
<td>A2</td>
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<td>+</td>
<td>−</td>
<td>15</td>
<td>14</td>
<td>12</td>
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<tr>
<td>B2</td>
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<td>−</td>
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<td>10</td>
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<td>10</td>
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<tr>
<td>D2</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>10</td>
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</table>

* Groups A1--D1 were in the first experiment; Groups A2--D2 were in the second identical experiment. RT = radiation; ATS = anti-rat thymocyte serum; NHS = normal horse serum; IC = intracerebral.
† Number of animals surviving preparation. The 10 preparation deaths were on Day 1 or 2 and were due to anesthetic overdose or respiratory arrest during IC inoculation.

Fig. 1. Mortality distribution of adult Fischer rats immunosuppressed with anti-rat thymocyte serum (ATS), radiation (RT), or ATS and RT. In both experiments, 86% of Group A (ATS and RT) rats died 13 to 27 days after intracerebral inoculation of the D-54 MG tumor.

Fig. 2. Mortality distribution of newborn Fischer rats immunosuppressed with anti-rat thymocyte serum. Reproducible mortality of 87%, 100%, and 100% of animals 18 to 34 days after intracerebral inoculation of D-54 MG tumor was demonstrated.
Intracerebral glioma transplant in immunosuppressed rats

**TABLE 2**

<p>| Experimen- | D-54 MG Injection | No. of Rats | Spontaneous Deaths | Large IC Tumors |</p>
<table>
<thead>
<tr>
<th>Tal Group</th>
<th>ATS</th>
<th>NHS</th>
<th>Dose</th>
<th>On Day:</th>
<th>Initial</th>
<th>Surviving†</th>
<th>No.</th>
<th>%</th>
<th>Days</th>
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<td>A</td>
<td>+</td>
<td>-</td>
<td>5 μl</td>
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<td>11/11‡</td>
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<tr>
<td>B</td>
<td>-</td>
<td>+</td>
<td>5 μl</td>
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<td>10</td>
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<td>0</td>
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<td>0</td>
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<tr>
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<td>+</td>
<td>-</td>
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<td>7</td>
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<td>15</td>
<td>13/15</td>
<td>87</td>
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<td>10</td>
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<tr>
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<td>-</td>
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<td>20</td>
<td>20</td>
<td>13</td>
<td>13/13</td>
<td>100</td>
<td>18-28</td>
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<tr>
<td>F</td>
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<td>10 μl</td>
<td>20</td>
<td>10</td>
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<td>0</td>
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</tbody>
</table>

* ATS = anti-rat thymocyte serum; NHS = normal horse serum; IC = intracerebral.
† Twenty-one animals died prior to tumor inoculation because of starvation secondary to foster-mother neglect or cannibalism.
‡ Three animals were withdrawn from the experiment on Day 15 for Evans blue dye or horseradish peroxidase injections. All had intracerebral tumors.

**Morphological Results**

Sixty-one tumors were seen in the 144 brains examined, and 57 (93%) of these tumors were accompanied by mass effect and were considered to be the cause of death. Tumors were well circumscribed, with distinct borders (Fig. 3). Large lesions frequently invaded or replaced the ventricles. The tumors contained solid sheets of polygonal cells that had convoluted nuclei and prominent nucleoli (Fig. 4). Mitoses were abundant and there were occasional multinucleate giant cells. Areas of serpentine necrosis bordered by zones of concentrated nuclei were seen. The tumors were well vascularized, but there was no endothelial proliferation. Lymphocytic infiltration was minimal or absent in all brains.

**Karyotype**

The number of chromosomes in each cell was determined for 50 cells in the direct preparation of one rat tumor, and for 50 cells in the 72-hour culture preparation of a second rat tumor. The range for all 100 cells was 66 to 73 chromosomes per cell, with 80 of 100 cells falling within a broad mode of 68 to 71 chromosomes.

Nine modal cells were karyotyped. All showed the same general chromosome distribution as has been seen

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*Fig. 3. Horizontal whole-mount section of rat brain showing D-54 MG tumor growing intracerebrally. H & E.*

*Fig. 4. Photomicrograph of D-54 MG tumor obtained from a rat brain showing sheets of polygonal cells with convoluted nuclei and prominent nucleoli. Numerous mitoses, including a tripolar mitotic figure, are seen. H & E, × 270.*
in D-54 MG tumors and contained 17 of the 18 markers previously reported in that cell line
(Fig. 5). The rat-grown cells differed consistently from the cultured line in that one No. 3 chromosome and one No. 5 chromosome were replaced by markers derived from these chromosomes, Marker IX was absent, and two No. 20 and one or two No. 21 chromosomes were missing. Three new marker types were sometimes observed: 15q-, 19q+, and one to three small unidentified fragments. Other sporadic changes were the loss of one Marker III, loss of one No. 5 chromosome, the replacement of one Marker V by a new marker type derived from it, and the loss of one No. 10 chromosome.

Discussion

New forms of cancer therapy should be assessed in animal models to evaluate efficacy and predict toxicity before use in a clinical setting. We report here the characterization of a transplantable tumor model in which the human tumor D-54 MG was grown in the brains of immunosuppressed Fischer rats. The D-54 MG cell line was chosen for a number of reasons. It is a malignant, highly tumorigenic neoplasm in nude mice, which makes it potentially suitable for transplant human neoplastic tissue in immunocompetent rodents. Numerous investigators have attempted to establish human xenografts in the anterior chamber of the eye or in the brain; however, growth is highly variable and occurs only after a long latency. We considered the use of athymic nude rats; however, at least three recent reports have demonstrated low rates of initial growth and high rates of subsequent regression as compared to athymic mice. We chose to immunosuppress both newborn and adult rats with ATS and radiation, individually or in combination. The
Intracerebral glioma transplant in immunosuppressed rats

optimal method (ATS to newborn rats) was adapted from the technique of van Steenis and van Wezel,\textsuperscript{23} who gave these injections subcutaneously to newborn rats to increase tumorigenicity of HeLa cells.

It is possible that the immunosuppression necessary to obtain human glioma growth in a heterologous species would alter the predictability or correlation with therapeutic responsiveness in human glioma patients. Although the mechanism of immunosuppression may be different between rats with ATS and human glioma patients, Mahaley, \textit{et al.},\textsuperscript{16} have demonstrated impaired cell-mediated immune response in 59 patients with malignant gliomas prior to surgery (for a literature review, see Brooks and Roszman\textsuperscript{3}). Other mainstays of human glioma treatment—radiation, steroids, and chemotherapy—also impair immune function.

The main value of an animal brain-tumor model is its ability to predict events in patients with brain tumors. Limitations of the applicability of our immunosuppressed rat glioma model to humans include 1) its transplantable (rather than autochthonous) character, which makes it developmentally less comparable to a human tumor, and 2) the size of the Fischer 344 rat, which precludes the evaluation of surgical debulking or computerized tomography. However, if in subsequent years it is determined that effective methods of diagnosis and treatment in this model are also clinically effective, it will become a valuable tool for the preclinical investigation of new approaches to human gliomas in areas such as immunodiagnosis, immunotherapy, and chemotherapy.

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\textbf{References}

mice and treatment with procarbazine, 1,3-bis(2-chloroethyl)-1-nitrosourea, aziridinyl benzoquinone, and cisplatinum. Neurosurgery 12:672–677, 1983


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