Development of a Xenograft Glioma Model in Mouse Brain

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ABSTRACT

Xenograft intracerebral glioma models have been developed in normal mice by growing the rat C6 glioma in either adult or neonatal mouse brains. Using this tumor line it was possible to grow discrete intracerebral gliomas in either CBA or AKR adult mice or neonatal mice. The size of the tumor mass and length of survival was directly related to the number of tumor cells injected and the time after implantation. To obtain localized intracranial tumor growth cells were suspended in a 1% agarose solution before implantation. Following injection of 10⁵ cells into the frontal lobe of adult CBA or AKR mice, discrete tumor masses greater than 4 mm in diameter were obtained in 90% of animals at 14 days, and the largest tumors in adult mice occurred between 21 and 28 days after implantation. The tumor size following implantation of 10⁶ cells was significantly greater than with 10⁵ cells at 7 days (P < 0.05) and at 14 and 21 days (P < 0.01). Less than 60% of mice of BALB/c, RIII, or C57 black strains developed tumors greater than 4 mm diameter at 14 days after intracranial injection of 10⁶ C6 cells. Using neonatal mice it was found that when 10⁵ cells were injected intracranially tumors greater than 4 mm in diameter were developed in 14 of 15 animals within 2 weeks (CBA mice). Similar results were seen in the RIII, AKR, C57 black, and BALB/c strains. Longer growth periods resulted in larger tumors, up to 8 mm in diameter (6 of 10 animals at 20 days). The tumors in the neonatal animals were not as discrete as in the adult mice, and tumor often spread to the meninges and into the lateral ventricles. The tumor harvested from the brain had a cloning efficiency of 1.2 ± 0.4% (SD).

A panel of monoclonal antibodies was raised to the C6 glioma, and this was used to defined the margins of the tumor within the brain. The xenograft mouse models should prove useful for the study of the therapy of gliomas.

INTRODUCTION

The results of treatment of malignant gliomas of the brain are most unsatisfactory. Even the best results in malignant gliomas of high grade treated with surgery, radiation therapy, and bischloroethylur素rea systemic therapy report a median survival time of less than 1 year (1–3). An accurate and easily reproducible animal model of the tumor is essential if there are to be significant improvements in patient survival. Although many tumor systems have been developed for the study of glioma, none of these is not entirely representative of the clinical situation, and there are difficulties in housing and handling these mice. Ependymoblastoma tumor has been used as an implantable intracerebral tumor model in normal mice (10), but this is a rare tumor in humans (4).

We have developed an easily reproducible xenograft mouse glioma brain model using the C6 rat glioma in both neonatal and adult mouse brains. The C6 glioma cell line was originally produced by i.v. injection of N-nitrosomethylurea into male Wistar rats (13). The features of this cell line have been described in detail previously (13–16). A panel of monoclonal antibodies was raised to the C6 tumor, and these were used to define the brain-tumor border. These glioma tumor models and the monoclonal antibodies should prove useful for the evaluation of new tumor therapies.

MATERIALS AND METHODS

Cells. The C6 glioma cell line was obtained from the American Type Culture Collection, and the cells were grown in RPMI 1640 medium supplemented with 10% FCS (Commonwealth Serum Laboratories, Parkville, Australia). Cells were harvested during the log phase of growth.

Intracranial Implantation of C6 Glioma. Adult mice from 6–8 weeks of age and neonatal mice less than 48 h old obtained from the Walter and Eliza Hall Institute animal colony (Melbourne, Australia) were used. The strains investigated in this study were CBA, BALB/c, AKR, C57 black, and RIII. To establish intracranial gliomas in mice the cells were suspended in a solution of double strength RPMI 1640 containing 1% agarose and kept at room temperature until injected.

Adult mice were anesthetized by penrose inhalation, and a 1-cm midline scalp incision was made. If the coronal suture was fused, a burr hole was inserted with a 1-mm dental burr 1 mm in front of the coronal suture, 3 mm to the left of the midline. The tumor cell suspension (10 µl) was injected using a Hamilton syringe with a 27-gauge disposable needle, either through the burr hole or through the unfused coronal suture 3 mm to the left of the midline. The needle was covered by a plastic sleeve permitting an injection depth of 3.5 mm from the outer table of bone. The needle was withdrawn 30 s after injection of the cell suspension, and the bone hole was covered with sterile bone wax (Ethicon, Edin-
burgh, Scotland). The skin was closed with a skin clip (Autoclip; Becton, Dickinson and Company, Parsippany, NJ).

Neonatal mice were given injections of 5 μl of tumor suspension through the skin using a 27-gauge needle covered with a plastic sleeve to permit an injection depth of 3 mm from the skin. The injection was 1 mm caudal to the coronal suture, which could be visualized with a strong light, and 2 mm to the left of the midline. The needle was withdrawn 15 s after injection of the cell suspension, and light pressure was then applied to the injection site for a further 15 s.

Subcutaneous Implantation. Fifty adult mice (BALB/c, CBA, AKR, RII, C57 black strain) were given injections of 10^6 cells (25 μl) i.c. in the midline 5 mm posterior to the intercanthal line.

Subcutaneous and Intracerebral Implantation. Sixty adult CBA mice were given injections of 10^7 (25 μl) i.c. 1 cm posterior to the intercanthal line. Intracranial implantation of C6 cells (10^4, 10^5, 10^6 cells; 15 in each group) was performed 2 weeks after the s.c. injection.

Animals were sacrificed by cervical dislocation at various times up to 60 days after inoculation. Brains were removed, placed in 10% formaldehyde, and sectioned after adequate fixation using conventional histological procedures. Tumor diameter was measured on serial sections using a graticule (Micrometer; Leitz, Wetzlar, West Germany).

Monoclonal Antibodies. A set of monoclonal antibodies were raised to the rat C6 glioma cell line. BALB/c mice were given injections i.p. with 2.5 x 10^6 cells, an i.p. boost of 8 x 10^6 cells 21 days later, and a final i.v. boost of 10^6 cells after a further 8 days, with fusion 4 days later.

The fusion procedure was basically as described by Brodsky et al. (17), with a fusion ratio of 5 spleen lymphocytes to one X 63-Ag-8,653 hybridoma cell. The hybridoma medium (HAT medium) was RPMI 1640 containing 30% FCS,2 HAT [hypoxanthine (114 μg/ml):aminopterin (0.176 μg/ml):thymidine (3.9 μg/ml), and OPIG [oxalacetic acid (150 ng/ml):L-glutamine (150 μg/ml)]. RPMI 1640 and FCS were from Commonwealth Serum Laboratories, Parkville, Australia, and the chemicals were from Sigma Chemical Co., St. Louis, MO. One hundred μl of the fusion mixture containing 10^6 myeloma cells were plated out into each of 384 6-mm tissue culture wells (A/S Nunc, Kamstrup, Denmark) with 10^6 BALB/c mouse thymocytes per well, fed with a further 100 μl of HAT medium after 3 days, and then fed twice weekly by removal of 100 μl of spent medium and replaced by fresh HAT medium. Hybrid colonies appeared after 11 days, and supernatants from hybrid-containing wells were screened for the presence of mouse antibody.

The screening assay was a solid phase enzyme-linked immunosorbent assay against C6 cells grown to subconfluency in microtitre trays and fixed with 0.0025% glutaraldehyde in phosphate-buffered saline, 10 min at room temperature. Fifty μl of supernatant from wells containing hybrid colonies were added to assay plate wells containing fixed C6 cells and were incubated for 30 min at 37°C. The wells were washed 3 times with PBS containing 0.05% Tween-20 and incubated for 30 min at 37°C with 50 μl of a 1/50 dilution of peroxidase-conjugated rabbit-anti mouse immunoglobulins (P260, Dakopatts A/S, Denmark). After 3 washes, 100 μl of a freshly prepared aqueous solution of 0.02% w/v o-phenylenediamine and 0.05% H2O2 were added to each well and allowed to react for 10 min at room temperature, and the reaction was stopped by the addition of 25 μl of 8 N H4SO4. Development of a brown color indicated the presence of mouse antibody in the hybridoma supernatant, and these colonies were scored as positive. Such colonies were expanded and cloned twice by limiting dilution on feeder layers of mouse thymocyte cells, 10^6 per microtitre well. Hybrids were grown up after second cloning; the monoclonal antibodies were isolated by an enzyme immunoassay (18) and screened by flow cytometry on the C6 tumor cell and by immunofluorescence and immunohistochemistry on frozen sections of rat and mouse tissue.

**Flow Cytometry.** C6 glioma cells were harvested in log phase growth using mEDTA. Aliquots of 10^6 cells were reacted with 0.5 ml control supernatant or LMM 25, 27, or 28 monoclonal antibody supernatants for 1 h at 4°C. The cells were washed with PBS containing 1% FCS and 0.5% sodium azide and were incubated for 30 min at 4°C with a 1/40 dilution of fluorescein isothiocyanate-conjugated rabbit-anti mouse immunoglobulin (Dakopatts A/S, Denmark). After a further wash, the cells were resuspended in 2 ml of PBS-FCS-azide and analyzed for cell surface staining on an Ortho System 50 flow cytometer (Ortho Diagnostic Systems, Westwood, MA).

**In Vitro Studies.** Gliomas were harvested from mouse brains 16 days after intracerebral inoculation of 10^6 cells. A single cell suspension was prepared by trypsinization and passage through a sieve (size 60 mesh). The viable cells were counted using eosin exclusion, and 200 to 2000 cells per dish were cultured in T25 flasks in RPMI 1640 with 10% FCS. After 7 days, colonies (greater than 50 cells) were counted.

**Statistical Analysis.** Data were analyzed using a 2-tailed Student's t-test to test the hypothesis that mean values in each group were equal. A P-value of less than 0.05 was considered significant.

**RESULTS**

**Adult Mice.** When 10^7 cells were injected into CBA adult mice, 20 of 20 animals developed tumors 4 mm in diameter or greater within 14 days, and 90% of the animals were dead by 14 days (Fig. 1A). When 10^6 cells were implanted, 9 of 10 animals had tumors larger than 4 cm in diameter at 14 days, and 90% of the animals died by 24 days (Table 1; Figs. 1A and 2A). The growth of the intracerebral tumor following injection of 10^6 cells is shown in Fig. 3. It can be seen that the tumor progressively enlarges without macroscopic central necrosis and with a distinct tumor-blood margin. There is considerable ventricular distortion in those brains harboring large tumors. There was no significant difference in the tumor size between animals given injections of 10^7 cells after intracerebral inoculation of 10^6 cells. A single cell suspension was prepared by trypsinization and passage through a sieve (size 60 mesh). The viable cells were counted using eosin exclusion, and 200 to 2000 cells per dish were cultured in T25 flasks in RPMI 1640 with 10% FCS. After 7 days, colonies (greater than 50 cells) were counted.

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**Table 1**

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* Less than 5% of animals survived to this time.

**Table 2**

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* No animals survived to this time.

**A XENOGRAFT GLIOMA MODEL IN MOUSE BRAIN**

*The abbreviations used are: FCS, fetal calf serum; PBS, phosphate-buffered saline (mouse toxicity, pH 7.4); HAT, hypoxanthine-aminopterin-thymidine; MNU, methyl nitrosourea.*

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A XENOGRAFT GLIOMA MODEL IN MOUSE BRAIN

Fig. 1. In vivo intracerebral growth of C6 glioma in mice. Tumor size (mm) is shown following intracerebral implantation of C6 tumor cells (10^6, 10^7, 10^8, 10^9, 10^10) into adult CBA mice (A) and neonatal mice (B). The ordinate shows the mean size of the tumors, and the abscissa shows the time (days) following implantation (n = 4-10 for each group). Bars, SE. •, 10^6 cells injected; ▲, 10^8 cells injected; O, 10^9 cells injected; △, 10^10 cells injected; ■, 10^11 cells injected.

Fig. 2. Survival of mice following intracerebral inoculation with C6 glioma. Survival of adult CBA mice (A) and neonatal mice (B) following intracerebral inoculation of C6 glioma cells (10^6, 10^7, 10^8, 10^9, 10^10) (n = 4-15 for each group). •, 10^3 cells injected; ▲, 10^6 cells injected; O, 10^7 cells injected; △, 10^8 cells injected; ■, 10^9 cells injected.

Fig. 3. Photomicrograph of coronal sections of brain showing the intracerebral tumor at 7 days (a), 14 days (b), 21 days (c), and 28 days (d) after inoculations of 10^6 C6 glioma cells into adult CBA mice. Bar, 2 mm.

The largest tumors were 6.8 mm in diameter, and these occurred in CBA mice 21–28 days after injection with 10^7 cells. The sex of the animal did not influence either the rate of growth of the tumor or the length of survival following injection (data not shown).

Similar results were obtained following implantation of C6 tumor into 50 mice of AKR strain. However, tumor growth was less reliable in the BALB/c, RIIL, and C57 black strains. At 14 days after 10^7 cells were implanted, 5 of 10 BALB/c, 6 of 10 RIIL, and 4 of 10 C57 black mice had tumors greater than 4 cm. At 21 days after injection of 10^6 cells 3 of 5 BALB/c, 3 of 5 RIIL, and 2 of 5 C57 black mice had tumors greater than 4 mm in diameter, and at 28 days 3 of 5 Balb/c, 2 of 5 RIIL, and 2 of 5 C57 black mice had tumors 4 mm in diameter or larger (Table 2). All these tumors had significant lymphocytic infiltration at the margins of the tumor.

Neonatal Mice. When 10^5 cells were injected intracranially...
tumors were not as discrete as those found in the adult mouse occurred in 14 of 15 animals within 14 days (Table 3). These tumors had sizes following implantation of $10^5$ cells was smaller than 4 mm in diameter at 14 days, but no tumor (0 of 30) reached this size if $10^2$ cells were implanted. No tumors developed after implantation of $10^3$ cells, mitotic figures (1-2 per high power field), and vascular proliferation. The tumor was highly vascular, and necrosis occurred only occasionally around residual agarose in the center of the tumor.

There was a discrete border between the growing edge of the tumor (Fig. 4) and the surrounding brain, although in some areas tumor cells were seen to be invading the brain (Fig. 4).

If $10^5$ or $10^6$ cells were injected there was only slight lymphocytic infiltration, which was evident on the periphery of the tumor. When smaller numbers of cells were implanted there was greater lymphocytic infiltration around the tumor and within the tumor mass.

Identification of Tumor Margins Using Monoclonal Antibody. There were 5 hybridomas produced which secreted monoclonal antibody directed at the C6 glioma cell line. The fluorescence profiles from the flow cytometer (data not shown) show that each of the 3 monoclonal antibodies (LMM 25, LMM 26, and LMM 27) react strongly and uniformly with unfixed C6 cells into neonatal CBA mice, tumors greater than 4 mm in diameter occurred in 14 of 15 animals within 14 days (Table 3). These tumors were not as discrete as those found in the adult mouse brain model, and there was often extensive spread to the meninges and into the lateral ventricles. Longer growth periods resulted in larger tumors, up to 7.7 mm in diameter (6 of 10 animals at 20 days). Following implantation of $10^6$ cells, 6 of 10 animals had tumors larger than 4 mm in diameter at 14 days, but no tumor (0 of 30) reached this size if $10^2$ cells were implanted. No tumors developed after implantation of $10^3$ cells (0 of 15). The tumor size following implantation of $10^6$ cells was significantly greater compared with $10^4$ or $10^5$ cells at 7 (P < 0.05), 14, and 21 days (P < 0.01) (Fig. 18). Injection of $10^6$ cells produced tumors larger than 4 mm in diameter at 14 days in 5 of 5 animals. However no animal survived longer than 17 days from the time of injection of $10^6$ cells, whereas 50% of animals given injections of $10^5$ cells survived longer than 20 days (Fig. 28).

Fig. 1 shows that following implantation of $10^4$, $10^5$, or $10^6$ cells the tumor size was larger in neonatal mouse brains than in adult brains at times from 7 to 14 days ($10^6$ cells), 7 to 28 days ($10^5$ cells), or 14 to 42 days ($10^4$ cells). After implantation of $10^3$ cells the tumor was larger in neonatal mouse brains from 7 to 42 days (P < 0.05).

Other strains of mice were tested; similar growth patterns were seen in neonatal RIII, C57 black, AKR, and BALB/c mice (at least 45 mice of each strain were given injections).

Five adult mice died within 24 h of the injection in the first 100 mice given injections. Perioperative mortality was 2% for the remainder of the animals given injections. The brains were removed from 88% of those animals that died in the perioperative period and were fixed in formalin. Three animals had intracerebral hematomas, and one had an extracerebral hematoma. The major cause of early death was thought to be due to anesthetic complications which occurred especially early in the series, as there was some difficulty in judging the appropriate depth of anesthesia.

A complete autopsy of 25 animals that died as a result of their intracranial tumor was performed, and no metastatic tumors were found. However, in the mice that were given injections in the neonatal period, there was considerable extra cerebral spread of the tumor that invaded the bones of the cranial vault and the pericranium.

Subcutaneous and Intracranial Implantation. Previous s.c. inoculation of $10^7$ C6 glioma cells did not affect the growth of intracerebral tumors in adult CBA mice following implantation of $10^5$, $10^6$, and $10^7$ cells. However the size of intracranial tumor that developed after implantation of $10^6$ cells was significantly smaller (P < 0.05) at 7, 14, and 21 days compared with tumors that developed if there had been no previous s.c. inoculation.

Subcutaneous Implantation. There was no growth of tumor following s.c. implantation of the cells in either the adult or neonatal mice.

Histology of Tumor. The histological appearance of the tumor was of a high grade malignant astrocytoma with multinucleated cells, mitotic figures (1-2 per high power field), and vascular proliferation. The tumor was highly vascular, and necrosis occurred only occasionally around residual agarose in the center of the tumor.
in suspension, indicating that they bind to cell surface antigens. None of the antibodies stained normal human, mouse, or rat brain parenchyma. LMM 25 stained normal rat colon and lung, but LMM 27 and LMM 28 were negative on all normal rat tissue tested (including colon, lung, liver, and spleen).

Frozen sections of CBA mouse brains containing growing C₆ tumors were stained by either 2 layer immunofluorescence or immunoperoxidase methods. Fig. 5 shows a tumor margin stained by LMM 27 immunohistochemistry. There was strong staining of all tumor cells, which is obvious by their large pale nuclei. Areas of normal brain beyond the tumor margin are unstained, but there were a number of small islands and projections of tumor cells beyond the margin, all of which stained strongly with this antibody.

**DISCUSSION**

We have shown that the rat C₆ glioma can be reliably grown in a xenograft mouse model. It is particularly useful because it grows in both neonatal and adult mice, in a localized region of the brain and in a reproducible manner. Growth of the glioma can be detected by morphological criteria, in vitro cloning, and by a monoclonal antibody that only detects the glioma and not the normal brain parenchyma. A disadvantage of the model is that it is based on a transplantable cell line rather than an induced tumor. The histology of the C₆ tumor in this model was similar to a high grade astrocytoma in humans. Although the tumor has a relatively discrete edge macroscopically, it did resemble the human glioma in that there was marked microscopic invasion of the tumor into the surrounding brain, which was confirmed by monoclonal antibody staining.

When 10⁶ cells were injected into the frontal lobe discrete tumor masses greater than 4 mm in diameter were obtained in 90% of animals at 14 days. Animals given injections of 10⁷ cells died with tumors less than 4.5 mm in diameter. The largest tumors in adult mice were obtained between 21 and 28 days after implantation of 10⁶ cells. However, as 80% of the animals with this size inoculum died after 20 days, the most appropriate time to utilize the animals harboring the tumor is between 14 and 18 days after implantation of 10⁶ cells. In adult mice, tumors larger than 6.2 mm diameter were not compatible with survival. To obtain localized tumor growth it was essential to suspend the cells in a 1% agarose solution before implantation. Using this tumor line and CBA adult mice it was possible to predict the size of the tumor mass and length of animal survival based on the number of cells injected and the time after implantation. In neonatal animals larger tumors (up to 7 mm in diameter) were obtained at the time of death, as the cranial vault was able to expand due to the unfused cranial sutures.

The tumor was able to grow intracranially but not s.c. perhaps because the brain is an immunologically privileged organ (19–28). Nevertheless lymphocytes specifically reactive to glioma cells have been shown in the peripheral circulation (29, 30), and lymphocytic infiltration has been demonstrated around human gliomas (31, 32). MNU-induced tumors have been shown to be highly immunogenic in vivo (14, 33), although transplacental ethylnitrosourea-induced tumors are capable of only weaker rejection responses (34). The reason that the C₆ tumor grew in the CBA and AKR adult mice and not as reliably in other strains may be related to the histocompatibility receptors, as the CBA and AKR mice have the same major histocompatibility type (H-2k).

If the adult mice were immunized with C₆ cells before intracerebral implantation there was no difference in tumor growth at high levels of tumor inoculation (10⁶ to 10⁷ cells), but growth of...
the tumor after implantation of $10^4$ cells was impaired. The C6 tumor has been used to develop a homograft in vivo model in rats (35). As shown previously the tumor grows to a larger size in a syngeneic host (male Wistar rats) at low doses ($10^4$ cells) of intracerebral inoculation, but there was no difference in the growth rate at higher dose ($10^5-10^7$ cells) (36) (Table 4). These results indicate that there is a small immunological reaction which is sufficient to affect the growth of the tumor after small doses of intracerebral inocula, but it is not sufficient to affect the growth of the tumor mass if larger numbers of cells are implanted.

The growth of human xenograft tumors grown in normal animals has been described by Greene and Arnold (37), and Greene (38). However, other authors have not been able to reproduce these results (39). Although the brain is a relatively immunologically privileged site, injection of tumor cells reduces the immunological protection due to the inevitable trauma to the brain on implantation. It is possible that the factors allowing the growth of the tumor in this model were that high concentrations of cells suspended in an agarose medium resulted in a critical growth of the tumor in this model were that high concentrations of cells suspended in an agarose medium resulted in a critical immunological protection due to the inevitable trauma to the brain during introduction of the tumor cells but a substantial benefit in that the position and size of the tumor can be predicted accurately. Another implantable glioma model in the normal mouse is the ependymoblastoma, which was initially induced by the chemical carcinogen 3-methylcholanthrene (40-42). However not only is the ependymoblastoma a rare tumor in humans, but the relevance of the mouse ependymoblastoma to the human disease is unclear (4) because the mouse cells lack many of the ultrastructural characteristics of ependymal cells (45) and the cell proliferation kinetics are unlike those of human tumors (8).

A monoclonal antibody has been developed previously to the C6 rat glioma (46). Our antibody may be similar to that described previously, although the application of the antibody staining in tissue sections to exactly define the tumor margin of gliomas appears to be novel. It has been suggested previously (47) that monoclonal antibodies may be useful for the therapy of gliomas when coupled to chemical toxins, chemotherapy agents, or radionuclides, but a lack of specificity of the antibodies has been an important factor limiting its use in patients (47). The specificity of our antibodies should allow in vivo testing of these types of therapies using our xenograft tumor model.

This C6 glioma xenograft mouse model and monoclonal antibody should prove useful for the study of therapy of glioma tumors, as both the size and the position of the tumor can be judged accurately, and the monoclonal antibody clearly defines the tumor extension into the adjacent brain. In addition, the C6 glioma cell line is easily grown in tissue culture, maintenance stocks can be kept frozen for future use, good single cell suspensions can be prepared for accurate quantitative estimation of tumor inocula, and the presence and survival of tumor cells can be accurately monitored by in vitro culture or detection by monoclonal antibodies.

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