The Importance of a Syngeneic Glioma Implantation Model: Comparison of the F98 Cell Line in Fischer and Long-Evans Rats

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ABSTRACT

Objective: Certain characteristics are required in order to improve the validity of glioma animal models, an essential one being syngeneicity. The present study was designed to investigate the difference in inflammatory response evoked by implantation of the F98 cell line in its syngeneic host (the Fischer rat) compared to an allogeneic host (Long-Evans rat).

Methods: The F98 cell line was cultured in monolayer and stereotactically implanted in the right frontal lobe of 18 Fischer rats and 18 Long-Evans rats. Animals were sacrificed when symptomatic or at 30 days from the implantation, whichever came first. H&E staining and immunocytochemistry for GFAP, CD3, and CD45 were performed on the brain specimens. The difference in the number of cells labeling for CD3 between both rat strains was used as the basis for the comparison, and was correlated with the rate of tumor take between both groups.

Results: Tumor take was observed in 100% of the Fischer rats, compared to only 50% of the Long-Evans rats ($P = 0.0004$). Immune response was profuse in the Long-Evans rats, with a mean of 29.1 CD3-staining cells per high-power field, compared to 2.8 in the Fischer rats ($P < 0.01$).

Conclusion: In this study, the use of an allogeneic glioma model was associated with an important inflammatory response, presumably responsible for the low tumor-take observed in this group. We believe that future in vivo studies should ideally be carried out using syngeneic implantation models. The F98/Fischer model seems to be adequate for this purpose.

INTRODUCTION

Research in neuro-oncology traditionally requires adequate in vivo animal models, on which therapeutic strategies are to be tested before human trials can

be designed and proceed. Some of these models are derived from cultured cell lines that are implanted in the brain of the target animal. Certain determinants are considered important in improving the validity of such models.[1-4] Ideally, the implantation technique must be relatively non traumatic, with minimal brain parenchymal damage. The procedure must have a limited morbidity and no mortality for the host. Moreover, it should yield a tumor take rate close to 100%. The tumor masses that develop must have a growth rate that is relatively constant and reproducible among the different subjects implanted. The model should be developed in immunocompetent hosts rather than nude animals with no immune systems, in order to closely simulate the behavior of human tumors. The derived tumors should also be relatively resistant to chemotherapeutic agents and other treatment strategies, as are human malignant gliomas. Finally, we believe that an essential quality of any good implantation model is to be syngeneic in nature.

Immunogenic compatibility between a graft and its host can fall into three different categories. A xenograft implies that the cell line and the host are from two different species. An allograft represents a cell line implanted in an animal of the same species, but of a different strain. A syngeneic implantation requires that the cell line implanted had originally been derived from the host in which it is implanted, implying that they are of the same immunogenic origin.[5]

The theoretical and practical application for this last requirement stems from the need for the implanted tumor to be free of any impediment to its growth, other than the therapeutic agents that are to be tested on the system. As pointed out by many authors in recent articles, xenografts and allografts have the potential to evoke an immune response in the host against the tumor graft, leading potentially to tumor rejection.[1,5,6] This immune tumor rejection could be misinterpreted as a significant response to a therapeutic agent in the context of an efficacy study. This inflammatory reaction, triggered by a host-graft rejection, is presumed to be less important in a syngeneic model.

The F98 cell line is an anaplastic glioma with a minor sarcomatous component that was originally produced by a single N-ethyl-N-nitrosourea (ENU) injection to a 20-week pregnant Fischer rat.[1,7-9] The offspring developed a brain tumor that was harvested and maintained in culture. These cells have proven to be weakly immunogenic, with reports of successful implantation in cats.[1,10] The typically implanted animals develop infiltrative tumors that are typically very resistant to conventional treatment, with no significant response thus far reported to any tested agent.[1]

In this experiment, we sought to assess the difference in the evoked inflammatory reaction and in tumor take after the implantation of this cell line in its syngeneic host (the Fischer rat), compared to its implantation in an allogeneic system. The Long-Evans rat was chosen as the allograft subject. This rat strain has been used in our laboratory in the development of a blood-brain barrier disruption technique.[11] It was our hypothesis that the allogeneic model would be associated with a significant inflammatory response, which could invalidate its further use in neuro-oncologic research.

MATERIALS AND METHODS

Animal Groups

Two groups (group 1: Fischer, group 2: Long-Evans) consisting each of 18 adult male rats weighting approximately 200 grams were used in this study. Prior to the initiation of this study, approval was obtained from the institutional animal experiment review board.

Cell Culture
The F98 cell line was obtained from ATCC, and was grown in monolayer using a solution of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a mix of penicillin (100 UI/mL) and streptomycin (100 µg/mL). Cells were incubated at 37°C in a humidified environment with 5% CO_2 and propagated upon confluence, every 3 days.

The implantation solution was prepared by trypsinization of the cell culture followed by resuspension in a DMEM solution free of FBS. The solution was diluted to obtain a concentration of $5x10^5$ cells in a volume of 10 µL. A trypan blue exclusion test was performed to assess cell viability before implantation.

Implantation Technique
Anesthesia consisted of induction with inhalation of a mixture of oxygen with 5% halothane. Maintenance was obtained with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals were then mounted on a stereotactic frame. A midline scalp incision was performed, followed by exposure and identification of the bregma. A burr hole was drilled 1 mm anterior and 3 mm lateral to the right of the bregma, targeting the anterior right frontal lobe. A 25-microliter SGF syringe with a 27-gauge needle, previously attached to the frame and loaded with the cell suspension, was used to infuse the solution at a depth of 6 mm from the outer table of the skull. Infusion was done manually over a period of one minute, after which the syringe was slowly withdrawn. Bone wax was applied to close the burr hole and the scalp was closed with a continuous one-layer resorbable suture.

Post-procedure Monitoring
Animals were allowed to recover from the procedure, after which they were given food and water ad libitum. They were assessed clinically on a daily basis for the apparition of signs of raised intracranial pressure (lethargy, vomiting, cachexia) or focal neurological signs (hemiparesis). The subjects were weighted weekly and at the time of death. Sacrifice was carried by CO_2 inhalation at the time of apparition of clinical signs, or after a maximum period of 30 days had elapsed from the implantation procedure. Brains were retrieved immediately after sacrifice.

Specimen Processing
Upon retrieval, brain specimens were fixed in a formalin solution for 48 hours, after which they were cut in coronal plane in 2 mm-width slices and embedded in paraffin. The tissue samples were sectioned at 3 µm intervals and the resulting slides were stained with haematoxylin and eosin (H&E). Monoclonal antibody labeling was obtained for GFAP, to prove the glial nature of tumors, and for CD3 and CD45, to evaluate the presence of infiltrating intratumoral and peritumoral T and B lymphocytes, respectively. After deparaffinization and rehydration, a microwave antigen retrieval process was performed. Slides were placed in 0.1 mmol/L citrate buffer in a microwaveable pressure cooker and boiled in a 700-W microwave oven for 30 minutes. Sections were incubated with primary antibody (DakoCytomation Inc., Mississauga, Canada). A dilution of 1:15 was used for the GFAP antibody, 1:50 for CD3, and 1:800 for CD45. Biotinylated species-specific secondary antibodies were applied followed by an avidin-biotin amplification and peroxidase development.

Pathologic Analysis
Specific slides were scanned at low-power magnification to identify the

Table 1. Comparative Clinical Data for Both Groups

GROUP 1—LONG-EVANS RATS				
Rat	**Δ Weight (g)**	**Survival (days)**	**Symptoms**	**Tumor Take**
1	NA	10	Lethargy	+
2	NA	10	Lethargy	+
3	+22	15	Lethargy	+
4	+32	15	Lethargy	+
5	+12	15	Lethargy	+
6	+30	30	None	-
7	-60	11	Hemiplegia, lethargy	+
8	+63	30	None	-
9	+59	30	None	-
10	-28	11	Hemiplegia, lethargy	+
11	+9	30	None	-
12	-81	12	Hemiplegia, lethargy	+
13	-18	30	None	-
14	-6	30	None	-
15	-75	8	Lethargy, irregular breathing	+
16	+5	30	None	-
17	+20	30	None	-
18	+2	30	None	-
GROUP 2—FISCHER RATS				
Rat	**Δ Weight (g)**	**Survival (days)**	**Symptoms**	**Tumor Take**
19	-37	12	Hemiparesis, lethargy	+
20	NA	7	Found dead	+
21	-28	4	Lethargy, irregular breathing	+
22	-17	16	Lethargy, ataxia	+
23	-44	10	Lethargy	+
24	-2	16	Lethargy	+
25	-24	16	Hemiparesis, lethargy	+
26	-6	22	Hemiplegia, lethargy	+
27	-7	19	Hemiparesis, lethargy	+
28	-29	14	Hemiplegia, lethargy	+
29	-2	15	Found dead	+
30	+3	19	Hemiplegia, lethargy	+
31	+3	18	Hemiparesis, lethargy	+
32	-22	17	Lethargy, hemiplegia	+
33	-55	14	Hemiplegia, lethargy	+
34	-18	14	Lethargy, ataxia	+
35	-14	14	Lethargy	+
36	-19	15	Hemiplegia, lethargy	+

tumors, which were then examined at higher magnification. For CD3 and CD45, the peritumoral areas were assessed at high power (200X) and the number of staining cells was noted in 10 contiguous fields in the area of the highest density of labeling. The result was then reduced to a mean number per high-power field (HPF). To be deemed as an adequate labeling, staining had to be observed in the cytoplasmic membrane and inside the cytoplasm.

Table 2. Statistical Analysis for Tumor Take and CD3 Count

	Long-Evans	Fischer	*P*
Tumor take	50%	100%	0.0004*
CD3 (mean per HPF)	29.1	2.8	< 0.01†

*Fischer exact test
†Student *t* test for paired data and Wilcoxon signed rank test

Statistical Analysis
The difference in tumor take between the Fischer and the Long-Evans group was assessed using the Fischer exact test. The difference in labeled cell count for CD3 expression between the two groups of animals was evaluated using the Student *t* test for paired data, and the Wilcoxon signed rank test.

RESULTS

Clinical Data and Tumor Take
In the Long-Evans group, 9 out of 18 animals (50%) eventually developed signs of intracranial tumor (Table 1). These signs consisted of lethargy (n = 9), contralateral paresis (n = 3), and irregular breathing (n = 1), after a period ranging from 8 to 15 days (mean 12 days, median 11 days). Four of these animals showed some degree of weight loss. The remaining 9 animals (50%) lived for 30 days without developing any clinical signs. All but 2 animals gained weight in this group. All symptomatic animals had pathologic evidence of an expanding brain tumor (macroscopy and microscopy), whereas none of the asymptomatic subjects did.

All Fischer rats eventually developed signs of a brain mass and were sacrificed before the 30 days study limit (Table 1). The intervals from the implantation procedure to sacrifice ranged from 4 to 22 days (mean and median time 15 days). Clinical signs were as follows: lethargy (n = 16), contralateral paresis (n = 10), ataxia (n = 2), and irregular breathing (n = 1). Two animals were found dead in the morning; they were asymptomatic the day before. All but 3 animals lost weight during the observation period. All 18 animals had macroscopic and microscopic demonstration of a large brain tumor.

The comparative data for tumor take yielded a 100% rate in the Fischer group and a 50% rate in the Long-Evans group (Table 2). This difference was highly significant in favor of the Fischer group ($P = 0.0004$, Fischer exact test).

H&E Examination
In the Long-Evans group, all the rats that became symptomatic demonstrated large tumor masses, with distortion of the normal cerebral architecture and an important mass effect. Tumors were composed of a mixed population of cells, most presenting astrocytic characteristics and others showing a sarcomatous appearance. Large necrotic areas were seen inside the bulk of the neoplasm and many mitotic figures were observed. However, the parenchymal infiltration at tumor margin was very limited, and very few neoplastic cells were present in the adjacent brain parenchyma, permeating the peritumoral area. Perivascular infiltration was prominent at the edge of the tumor margin. The behavior expressed by these tumor cells was more reminiscent of cerebral metastatic disease than that of a glial neoplasm. In a significant number of specimens, there was an important infiltration of the tumor by

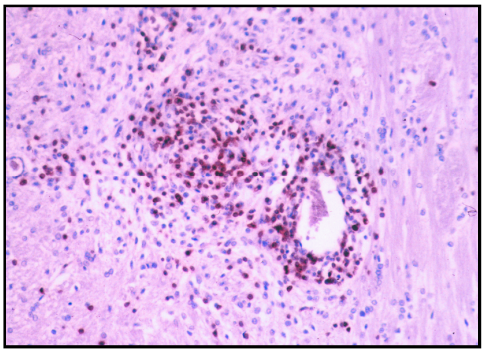

Figure 1. CD3 immunostaining, Long-Evans rat, 200X magnification. Lymphocytic infiltration was mainly observed around peritumoral capillaries, as clearly depicted in this illustration.

small round cells, which were morphologically similar to lymphocytes. The brain specimens of asymptomatic subjects showed no tumor take. Interestingly, in these animals, the implantation tract was clearly identifiable, with adjacent astrocytic reaction and infiltration by the same small round cell population.

The Fischer specimen examination revealed the presence of a brain tumor for every animal. Tumors were large, with a central necrotic core and numerous mitotic figures. Obvious distortion in the architecture was accompanied by important brain shift and mass effect. Vascular proliferative changes could be seen in some specimens. As in the other group, perivascular neoplastic infiltration was observed. However, in addition, individual tumor cells were seen permeating the adjacent brain parenchyma in all samples. The brain-tumor interface was not as distinct as it was in the Long-Evans group. The number of infiltrating lymphocyte-like cells was much less than in the Long-Evans group, with most specimens devoid of any.

GFAP Immunostaining

GFAP immunocytochemistry confirmed that a significant proportion of the neoplastic cells were of glial origin, with no

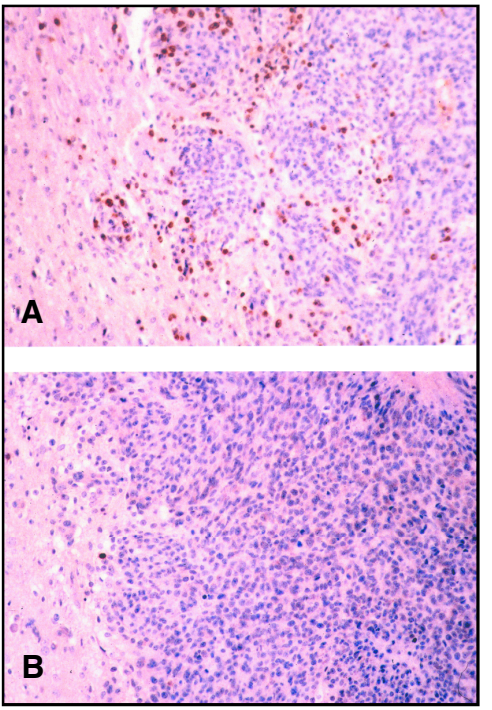

Figure 2. CD3 immunostaining, 200X magnification. Comparison between both groups. **A.** Long-Evans rat. Tumor margins show abundant CD3 staining, most likely representing an acute tumor graft rejection reaction in the setting of an allogeneic implantation. **B.** Fischer rat. Peritumoral region demonstrates scarce lymphocytic infiltration, with only a single cell staining in this example of a typical sample in the syngeneic setting.

difference in staining between the two groups.

CD3 Immunostaining

When positive, labeling for CD3 cells was seen at the periphery of the tumor and around adjacent capillaries (Figure 1). There was a significant difference in the amount of staining cells between both rat strains (Figure 2). In the Long-Evans group, a mean of 29.1 CD3 cells per HPF were present, whereas 2.8 staining cells per HPF were demonstrated in the Fischer specimens ($P < 0.01$, Student t test for paired data and Wilcoxon signed rank test) (Table 2).

In the Long-Evans specimens in whom there was no tumor take, CD3-

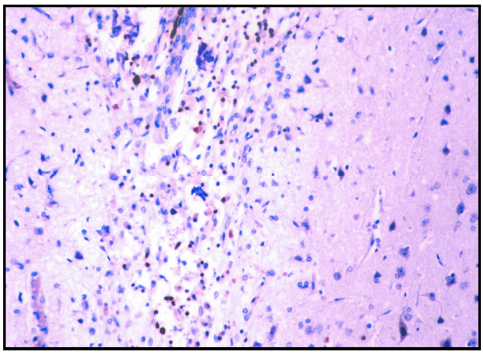

Figure 3. CD3 immunostaining, Long-Evans rat, 200X magnification. This picture illustrates an implantation tract in a Long-Evans rat in which no tumor take was observed. Disruption of the normal tissue architecture is observed with reactive gliosis. In addition, CD3 staining is clearly evident in and around the tract, presumably representing remnants of the inflammatory reaction that destroyed the tumor graft.

staining cells were demonstrated around the implantation tract (Figure 3).

CD45 Immunostaining

CD45 staining for B lymphocytes was scarce and insignificant in both groups.

DISCUSSION

Literature abounds with glioma models descriptions. The most widely used and reported model is the C6/Wistar model, originally described by Auer et al.[12] The C6 cell line was induced in rats after prolonged exposure to N-methylnitrosourea (MNU). The original host strain is unknown, and was assumed to be either the Wistar, the BDIX or the Sprague-Dawley rat.[1] Over time, it became traditional belief that the C6/Wistar model was an appropriate model and thus its use became widespread. A number of therapeutic strategies have been tested in pre-clinical in vivo setting using this model, with promising results. However, the translation of these results in human clinical studies has not met with the anticipated response rate. One of the reasons explaining this failure might be the inappropriateness of the model itself in allowing the evaluation of treatment strategies.[5] An ideal model should closely duplicate the human clinical condition. Thus, all the controllable factors permitting to emulate this condition as close as possible should be gathered. A recently published study by Parsa et al demonstrated unequivocally that the C6 cell line evokes a potent immune reaction when implanted in the Wistar rat, leading to spontaneous tumor cure in a majority of the specimens.[5] It is now current knowledge that the C6 cell line has no known syngeneic host.[1,5] Therefore, this cell line should not be considered in the design of an optimal implantation model.

In trying to circumvent the immunologic response to an allogeneic graft, and more closely simulate the human malignant glioma, many investigators explored the use of immunodeficient nude animal models.[6,13,14] However, this approach leads to another limitation, represented by the fact that most human tumors develop in immunocompetent patients. Interactions with the immune system, which probably plays an important role in any antitumoral strategies (and not only immunotherapy strategies), cannot be accounted for in these models. The presence of inactivated peritumoral lymphocytes has been well documented in human glioma samples.[15] The immune system is locally and systemically disrupted by soluble factors secreted by the tumor cells, and this reaction is integral to the development of a malignant glioma in humans.[15] Therefore, the presence of this amputated immune response is important in reproducing an adequate model emulating the human clinical situation. A syngeneic system is the most likely candidate to optimally model a malignant glioma. Few syngeneic models have been reported in the literature. The F98/Fischer is the most frequently cited,

and it has been characterized in some detail.[1,7-9] Recently, the CNS-1/Lewis model was described by Kruse and al, but it has not been extensively studied.[16]

In the present study, we demonstrated that even a relatively non immunogenic cell line like the F98 can lead to a significant inflammatory response when implanted in an allogeneic setting.[1] CD3-positive T-lymphocytes are the major vector of cellular immune response, and were significantly more abundant (29.1 vs 2.8 per HPF) in the tumors of Long-Evans rats compared to Fischer rats, the syngeneic host. B-lymphocytes response, which is not a significant component of the graft-rejection phenomenon, was insignificant in both groups.

We also hypothesize that the difference in tumor take (50% vs 100%) was directly related to this inflammatory reaction. Employing the same cell preparation and the exact same implantation technique in animals of similar weight and sex, the only variable between these two groups was the species. As a proof of principle, numerous T-lymphocytes were identified along the tumor implantation tract in most of the specimens devoid of tumor take (Figure 3).

Moreover, we believe that the immune compatibility of the host with the graft did influence the tumor characteristics in this study. The tumors had a more infiltrative pattern and emulated human malignant gliomas more closely after implantation in the syngeneic host (Fischer rats). This fact might be related to the immunologic reaction, which, we assume, was able to circumscribe and limit the expansion of the tumor in the Long-Evans group. The F98/Long-Evans actually behaves more like a metastasis model. Some authors have investigated the possibility of optimizing the existing models with different manipulations, with the goal of improving the infiltrative pattern in these tumors. With this goal in mind, Whittle et al experimented with mixed glioma cell lines without convincing results.[17] A possible solution to all these struggles might simply consist of using a syngeneic model.

The implantation procedure by itself might induce an exogenous inflammatory reaction, from the tissular traumatism inherent to the technique. Thus, an implantation system might not be an optimal way to produce the perfect glioma model. Transgenic models are probably the best overall models to emulate malignant gliomas and might eventually gain widespread recognition and use.[2,3] At the moment however, they still suffer from their intrinsic technical difficulties and high cost, which limit their use. Implantation models are relatively easy to produce. They are effective and predictable in inducing a reproducible tumor model and their availability is more widespread, requiring simple equipment.[4] In addition, we demonstrated in the present study that the immune response to the tissular trauma implantation is insufficient to significantly affect tumor growth when a careful technique is used in a syngeneic setting. This is illustrated by our results in the Fischer group.

CONCLUSION

The implantation of F98 cells in Long-Evans rat (allograft) leads to an important immune reaction compared to its use in Fischer rats (syngeneic model). Tumor take is suboptimal (50%) in the allogeneic model, and this is presumably due to the inflammatory response triggered. A syngeneic model is mandatory to evaluate further therapeutic strategies against malignant gliomas to minimize this inflammatory response. The F98/Fischer model appears adequate for this purpose, with a constant and predictable high rate of tumor take (100%), and an insignificant inflammatory reac-

tion triggered. Results from future preclinical studies using an allograft or xenograft implantation model should be interpreted cautiously, as they may be affected by significant bias from the model itself.

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