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Comparison of genetically engineered herpes simplex viruses for the treatment of brain tumors in a *scid* mouse model of human malignant glioma

(oncolytic viruses/gene therapy/tumor cell targeting)

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ABSTRACT **Genetically engineered viruses and viral genes inserted into retroviral vectors are increasingly being considered for experimental therapy of brain tumors. A primary target of these viruses and vectors is human gliomas, the most frequently occurring primary human brain tumor. To investigate the potential of genetically engineered herpes simplex viruses (HSVs) in the therapy of these tumors, we compared the attributes of two viruses, a recombinant from which the $\gamma_1$34.5 gene had been deleted (R3616) and a recombinant in which the $\gamma_1$34.5 gene had been interrupted by a stop codon (R4009). Previous studies have shown that these recombinants were completely devoid of the ability to multiply in the central nervous system of rodents. To pursue these studies, we developed a *scid* mouse glioma model. Tumor cell response (survival) for 10^3, 10^4, and 10^5 implanted MT539MG glioma cells was 38, 23, and 15 days, respectively. The results were as follows: (*i*) both R3616 and R4009 replicate and cause cytolysis in diverse glioma cell lines of murine and human origin *in vitro*, and (*ii*) in Winn-type assays 10^5 MT539MG cells coinoculated with R3616 or R4009 as compared to saline significantly prolonged survival in a dose-dependent fashion. Mice that received only tumor cells or the wild-type parent strain of the recombinants, HSV-1(F), died within 15 days. Survival was greatest with R4009. These experiments define both a model for screening oncolytic viruses and a genetically engineered virus of significant potential use as an oncolytic agent.**

Malignant gliomas are the most common primary intracranial malignant tumor, accounting for 30% of primary brain tumors in adults (1). The estimated tumor incidence in the United States is 14.7 per 100 thousand, resulting in ≈5000 new cases annually (2). In spite of aggressive surgical therapy, radiotherapy, and chemotherapy of patients with these tumors, the overall 5-year survival is <5.5%, and the median survival is ≈52 weeks, a figure that has remained virtually unchanged over the past 20 years (1–6). These abysmal survival rates have reinforced the need for new modalities of therapy.

Over the past several years, biomedical investigation has focused on the utilization of viruses as either direct therapeutics or for the transfer of genetic information, including the experimental therapy of brain tumors (7–16). For the experimental treatment of brain tumors, two approaches have predominated (5–12, 13–16). The first involves deliberate *in situ* inoculation of cells infected with a retrovirus expressing the herpes simplex virus 1 (HSV-1) thymidine kinase gene into the tumor mass followed by treatment with ganciclovir (GCV), an antiviral drug (8). The retrovirus is secreted from the inoculated cells and infects the tumor cells. GCV is selectively phosphorylated by the HSV-1 thymidine kinase to its monophosphate derivative and by cellular enzymes to a triphosphate derivative, which kills the tumor cells. This approach is currently undergoing clinical investigation at the National Institutes of Health. However, limitations of this approach include the quantity of nondividing cells that can be inoculated directly into the brain tumor, the relatively low yield of retroviruses, and the requirement for administration of GCV, a drug that has significant hematopoietic toxicity and does not penetrate the central nervous system to a great extent.

An alternative approach utilizes genetically engineered HSV. Among the mutants tested for this purpose were viruses lacking the thymidine kinase or ribonucleotide reductase gene or a genetically engineered virus lacking the $\gamma_1$34.5 gene constructed by one of our laboratories (16). Although some of the viruses tested to date prolonged the survival of tumor-bearing animals, none totally destroyed the tumor mass. Some of the deletion mutants tested, notably those that are thymidine kinase-negative, are potentially hazardous, since such viruses can cause encephalitis in animal models and are not treatable by drugs that depend on the viral thymidine kinase for their activity (17).

The interest in testing of $\gamma_1$34.5$^-$ viruses stems from studies on the function of the $\gamma_1$34.5 gene and the phenotype of these viruses carrying deletions and substitutions in that gene. The $\gamma_1$34.5 gene maps in the sequences flanking the long unique sequence and is present in two copies in the viral genome (18–20). Mutants lacking both $\gamma_1$34.5 genes (e.g., recombinant R3616) are apathogenic and fail to replicate in the central nervous system of mice (21). In cell culture, particularly in human fibroblasts and in the SK-N-SH human neuroblastoma cells, R3616 fails to prevent a stress response induced by the onset of viral DNA synthesis (22). In consequence, protein synthesis is totally and prematurely shut off, resulting in cell death and significantly reduced viral yields. Although R3616 possesses many of the properties desired for cancer therapy, its effectiveness may be limited because its host range is very restricted. Recent studies have shown that mutants carrying a stop codon in both copies of the $\gamma_1$34.5 gene (e.g., recombinant R4009) are equally avirulent after intracranial inoculation of mice and fail to preclude the stress response in SK-N-SH cells but, like wild-type virus, do not shut off protein synthesis prematurely in human foreskin fibroblasts (23). R4009 also multiplied better than R3616 in peripheral organs of mice (24). The objective of the studies described here was to establish a model of malignant glioma in mice and to compare the effec-

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Abbreviations: HSV, herpes simplex virus; pfu, plaque-forming unit(s); GCV, ganciclovir.

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tiveness of R3616 and R4009 in treatment of this tumor in the murine model.

MATERIALS AND METHODS

Cells and Viruses. HSV-1(F) is the prototype wild-type HSV-1 strain used in our laboratories (25). R3616 lacks 1000 bp from the coding domain of each copy of the $\gamma_1$34.5 gene. In R4009, a sequence containing a stop codon in all six frames was inserted into the $\gamma_1$34.5 gene after the 28th codon. All viruses were grown and titered in Vero cells as described (21, 22). The infected cells were disrupted by sonication, and the virus contained in the supernatant fluid after centrifugation at 1200 $\times$ *g* for 20 min was stored at −70°C.

The MT539MG glioma cell line was established from a spontaneous glioma from a VM/Dk (*H-2*^{b}) mouse (26, 27), and cells at passage 18 were kindly provided by D. D. Bigner (Duke University, Durham, NC). This cell line is composed of a mixed population of stellate, polygonal, and small rounded cells that are variably positive for glial fibrillary acidic protein, S-100 protein, and galactocerebroside (Fig. 1) and has a doubling time of 46.3 hr *in vitro*. For induction of tumors, MT539MG glioma cells were freshly harvested from logarithmic-phase growth cultures by brief exposure to buffers containing 0.25% trypsin and 0.53 mM EDTA. Cell inoculum consisted of the desired number of cells in 5 μl of serum-free tissue culture medium supplemented with 0.8% Methocel.

***scid* Mouse Model of Gliomas.** Intracerebral tumors were induced by allografting MT539MG cells into C.B-17 *scid/scid* (BALB/c background) mice. Mice were anesthetized [ketamine (20 mg/ml) plus xylazine (0.75 mg/ml) in saline administered intraperitoneally at 0.7 ml/10 g of body weight], a midline scalp incision (0.5–1 cm) was made, skin was reflected, and a 1-mm burr hole was made in the skull 1.5 mm to right of midline and 0.5–1.0 mm anterior to coronal suture. Tumor cells were loaded into a 250-μl Hamilton syringe fitted with a 30-gauge 0.5-inch needle, attached to a repeating dispenser, and mounted in a stereotaxic holder. The needle was inserted vertically through the burr hole to a depth of 2.5 mm as read from the vernier scale. After injection of 5 μl of cells into the right caudate nucleus, the needle was withdrawn after 30 sec, the burr hole was plugged with gel foam, and the skin was closed with sterile Michel 9-mm wound clips. Mice were returned to their sterile microisolator cages and maintained on autoclaved Lab Chow and sterile water, ad libitum. Studies with India ink have shown that the initial injection site in the caudate nucleus can be accurately retargeted 5–9 days later using the same burr hole and stereotaxic frame coordinates. All groups of mice contained 10 animals, and experiments were repeated a minimum of three times; where possible, survival data for identically treated groups were pooled.

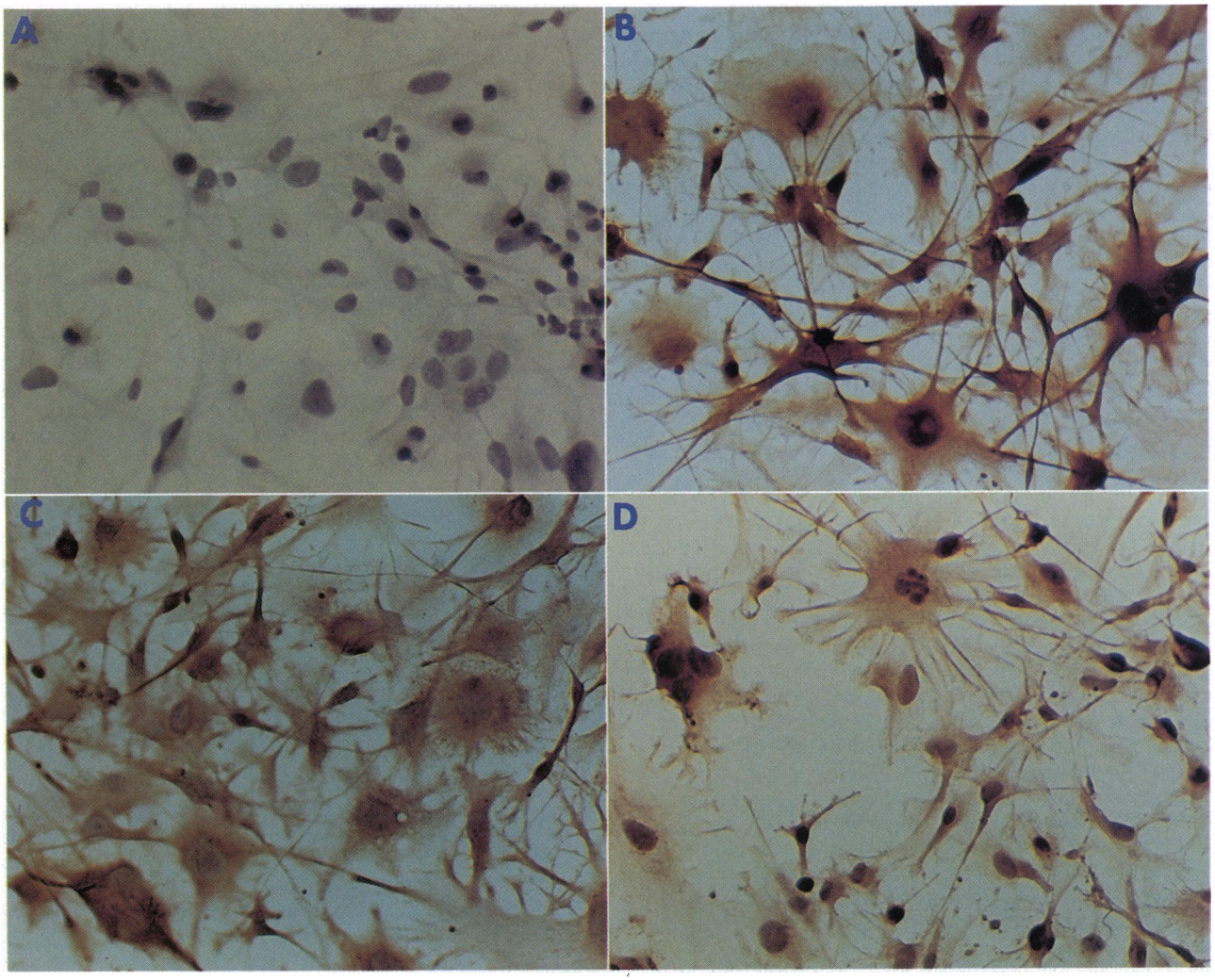

FIG. 1. Photomicrographs of MT539MG glioma cells grown in culture, fixed, and subjected to immunohistochemical analysis. Purified mouse IgG2a (*A*) was used as a nonspecific negative control. Primary antibodies were mouse monoclonal antibodies to glial fibrillry acid protein (*B*) or S100 protein (*C*) or rabbit anti-galactocerebroside antibody (*D*). Secondary antibodies to mouse or rabbit immunoglobulins were biotinylated, thus permitting development of peroxidase color reaction through the use of an avidin–biotin–horseradish complex and diaminobenzidine.

Therapeutic studies involved two approaches: (*i*) a Winn-type assay whereby virus at varying multiplicities of infection and a fixed quantity of tumor cells were simultaneously implanted into the brain, and (*ii*) a fixed number of tumor cells were implanted followed 72–96 hr later by intratumoral inoculation of virus at various multiplicities of infection.

Animal studies were done in accordance with guidelines for animal care by The University of Alabama at Birmingham Committee on Animal Care. All animal studies were performed in accordance with acceptable federal standards.

Tissue Harvesting, Isolation of Virus, Preparation, and Histopathology. Brain tissue was harvested from euthanized animals, fixed in 2% paraformaldehyde, embedded in paraffin, and serially sectioned at 10-μm intervals before, throughout, and after the tumor. Sections were routinely stained with hematoxylin/eosin and microscopically examined.

Brain tissue was harvested from euthanized animals to determine the presence of virus according to reported procedures (24).

Statistical Analyses. Kaplan–Meier survival data were analyzed with a computer software program (SURVCALC; Wiley, Sussex, England) to estimate significance of differences in the median survivals by the log rank and Peto–Wilcoxon nonparametric hypothesis tests. The χ^2 distribution was used to compute the probability, P, as determined at a significance level of <0.01.

RESULTS

***scid* Mouse Glioma Model.** Survival of mice is a function of the tumor burden inoculated intracerebrally. To establish a dose–response curve for tumor growth, survival of mice was calculated after inoculation of 10^5, 10^4, or 10^3 MT539MG cells. The results (Fig. 2*A*) show that the median survival for each of the three dosages was 16, 22, and 39 days, respectively. To obtain a rapid estimate of the relative efficacy of the genetically engineered HSV mutants, we selected standard dosages of 5–10 $\times$ 10^4 MT539MG cells per mouse inoculum for our studies. The histopathologic findings of treated and untreated tumors are discussed below.

Replication Competence of Engineered Viruses in Tumor Cells. Viral replication was measured in the MT539MG mouse glioma cell line exposed to 0.5 plaque-forming unit (pfu) of R3616, R4009, or HSV-1(F) per cell. The results (Fig. 3) show that all three viruses multiply in these cells. Similar results (data not shown) were obtained in studies on a human malignant glioma (U251MG).

Therapy of MT539MG Gliomas in *scid* Mice. Two series of experiments were done. In the first, tumor cells and R3616 or R4009 were admixed and inoculated simultaneously at various concentrations in a Winn-type assay. Alternatively, tumor cells were implanted and allowed to divide over 72–96 hr before intratumoral inoculation of virus at various multiplicities of infection. Mice receiving 10^5 MT539MG cells mixed with 2 $\times$ 10^5 or 10^8 pfu of R3616 survived for a median of 20 and 26 days, respectively (Fig. 2*B*). Peto–Wilcoxon estimations from Kaplan–Meier survival data of mice receiving either low or high doses of virus were significant (Fig. 2*B*), compared to that of mice implanted with MT539MG cells mixed with saline (P = 0.009 and 0.002, respectively). Median survival increased from 15 to 21 days (P = 0.0067, Peto–Wilcoxon analysis) if

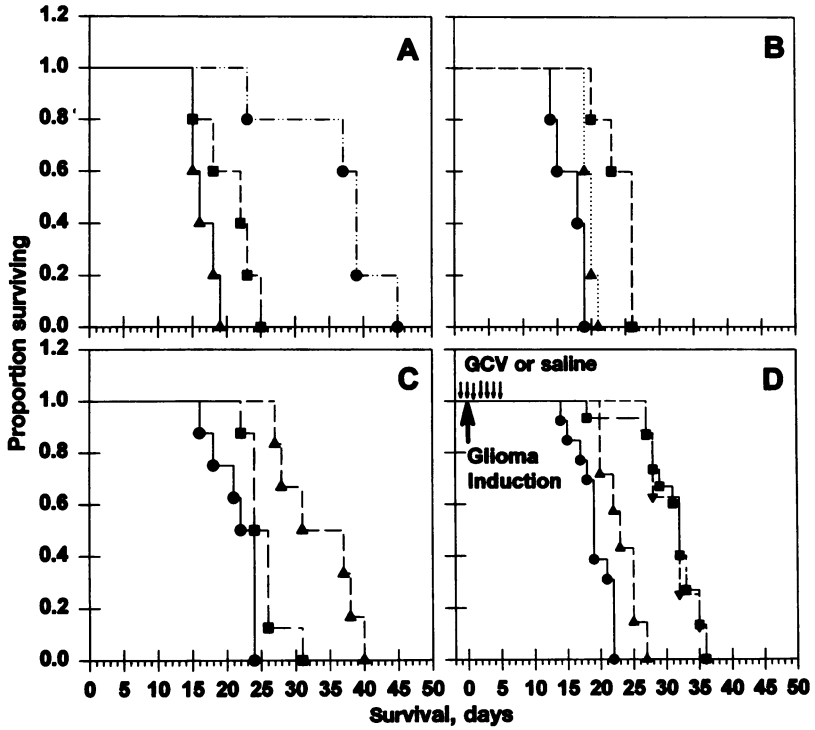

FIG. 2. (*A*) C.B-17 *scid/scid* mice, 6–12 weeks of age, were injected intracranially with graded numbers of MT539MG mouse glioma cells to determine an appropriate dose to use for experimental therapy evaluations. Mice that were moribund were killed, and the time of death was estimated to be 24 hr later. Deaths that occurred overnight were recorded. Brains were processed for histopathologic confirmation of cause of death. ●, 10^3 cells; ■, 10^4 cells; ▲, 10^5 cells. (*B*) C.B-17 *scid/scid* mice, 6–12 weeks of age, were injected (5 μl) intracranially with 10^5 MT539MG mouse glioma cells that had been mixed with saline (●) or 2.5 $\times$ 10^5 pfu (▲) or 10^6 pfu (■) of R3616 per ml. Deaths were either estimated or recorded as described. (*C*) MT539MG mouse glioma cells (5 $\times$ 10^4) were admixed with either saline (●) or 2.5 $\times$ 10^3 pfu (■) or 2.5 $\times$ 10^5 pfu (▲) of R4009 and injected (5 μl) intracranially in C.B-1.7 *scid* mice, 6–12 weeks of age. Deaths were either estimated or recorded as described. (*D*) C.B-17 *scid* mice, 6–12 weeks of age, received a single intracranial injection of 5 $\times$ 10^4 MT539MG cells mixed with saline or R4009 (5 $\times$ 10^5 pfu) on day 0. These mice received 7 daily intraperitoneal injections of saline or GCV at 25 or 50 mg/kg, beginning on day −1. Deaths were either estimated or recorded as described. ●, Saline (but not R4009) then 25–50 mg of GCV per kg (median survival = 19 days); ▲, R4009 then 50 mg of GCV per kg (median survival = 23 days); ▼, R4009 then 25 mg of GCV per kg (median survival = 32 days); ■, R4009 then saline (median survival = 32 days).

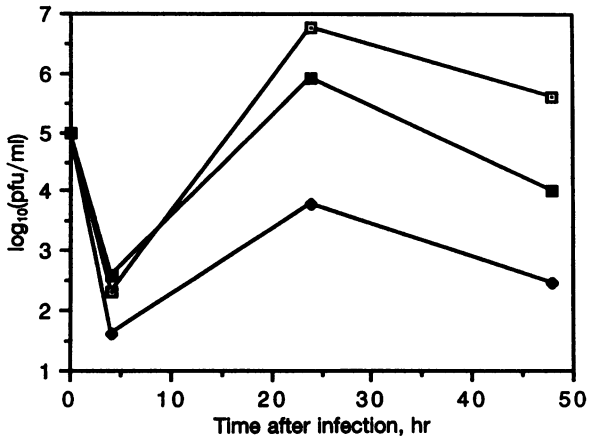

FIG. 3. Replication of wild-type and genetically engineered viruses in mouse glioma cells at different time periods. □, HSV-1(F); ■, R4009; ◆, R3616.

therapy was delayed until 72 hr after implantation; however, all animals died (data not shown).

In parallel experiments, 2.5×10^3 or 2.5×10^5 pfu of R4009 were mixed with 5×10^4 MT539MG tumor cells and implanted intracranially. Animals implanted with tumor cells mixed with saline died at a median of 22 days, whereas R4009-treated animals survived a median of 24 (P = not significant) and 30 ($P = 0.0006$) days for the low- and high-dose treatment groups, respectively (Fig. 2*C*).

If intratumoral injection of R4009 was delayed until 72 hr after tumor cell inoculation, 10^5 pfu of virus resulted in prolongation in median survival from 21 to 30 days ($P = 0.00011$); however, all animals subsequently died. We repeated this experiment at a dosage of 10^7 pfu 3 days after the establishment of tumor.

Intracranial injection of up to 2.5×10^6 pfu of either R3616 or R4009 into naive *scid* mice had no discernible effect over a 90-day observation interval. Intracranial injection of 10^4 pfu of HSV-1(F) caused death of all animals of fulminant encephalitis within 7 days after inoculation.

To demonstrate that increased survival of glioma-bearing animals was attributable to the mutant HSV, we sought to attenuate any protective (oncolytic) effects of the virus by administering either 25 mg (low dose) or 50 mg (high dose) of GCV per kg of body weight by intraperitoneal injection daily between −1 and 5 days relative to tumor-virus injection (day 0). GCV was reconstituted to 2 mg/ml in sterile saline for injection, and the appropriate dose was given in 0.2–0.6 ml

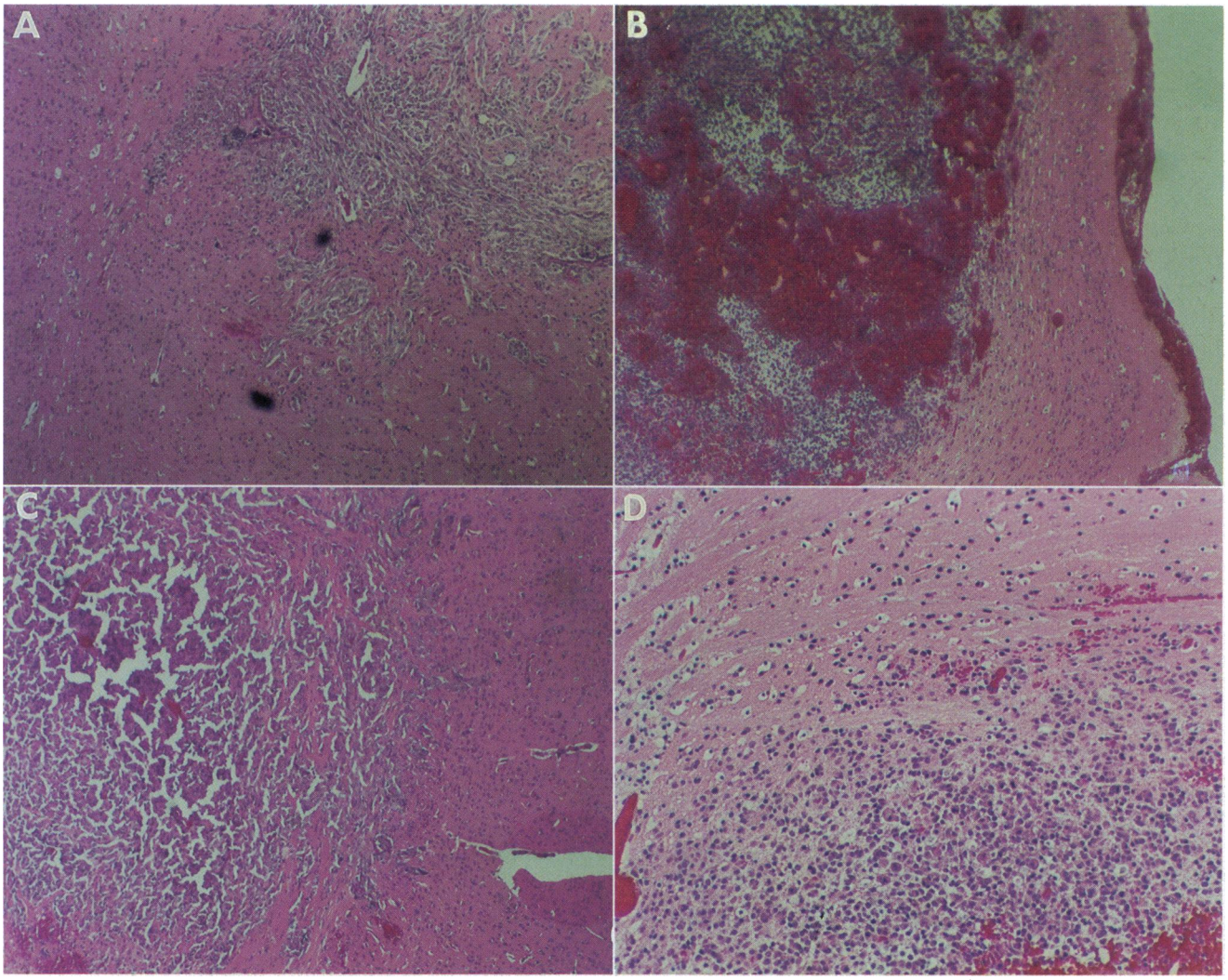

FIG. 4. Photomicrographs of MT539MG glioma induced in C.B-17 *scid/scid* mice subjected to euthanasia at 7 (*A*), 15 (*B*), or 26 (*C*) days posttumor induction. Tumors in *A* and *B* were untreated; the tumor in *C* had been injected at day 4 with 10^6 pfu of R3616. Gliomas were highly cellular with numerous mitoses, abundant capillaries with extravasation of erythrocytes, and areas of necrosis. (*D*) Tumor cells can be seen infiltrating the neuropil. Tumors could become quite extensive in size before animals were determined to be moribund and were euthanized. (*A–C*, ×45; *D*, ×95.)

intraperitoneally. Mice that received MT539MG glioma cells admixed with saline intracranially and treated with GCV had a median survival that was no different from all of the other trials (19 days). Likewise, mice that received MT539MG cells exposed to 10 pfu of R4009 virus per cell and given daily injections of saline survived significantly longer ($P < 0.01$), with a median survival of 32 days (Fig. 2*D*). Tumor bearers treated with low-dose GCV and R4009 survived as long as those animals treated with saline. In contrast, glioma-bearing mice that received high-dose GCV and R4009 survived only slightly longer than tumor bearers that were not given R4009 HSV. These data suggest a dose–response to GCV, whereby the higher dose partially reversed the protective effect of R4009 but the lower dose of GCV was not effective. As previously observed, progressively growing tumor was the cause of death in all of these mice; GCV has a LD_{10} in *scid* mice of 75 mg/kg, administered twice daily for 7 days (data not shown).

Studies on Brain Tissue Obtained at Necropsy. Brain tissue was harvested sequentially on days 15 and 30 and from long-term survivors to attempt to isolate HSV and perform histopathology as shown in Fig. 4. Specimens were obtained only from animals that received R4009. Attempts to explant HSV from brain tissue of all animals were uniformly unsuccessful.

DISCUSSION

The salient features of the studies described in this report are twofold. First, we have established an animal model that can be exploited for the rapid screening and evaluation of potential therapy of malignant gliomas. Since the sensitivity of the assay can be varied according to the number of tumor cells inoculated, it is possible to prolong survival with lower doses of cells and, therefore, provide a greater opportunity to demonstrate a tumoricidal effect of multiple treatments with lower doses of cells. We have elected to screen for suitable oncolytic viruses by raising the number of cells to the maximum that can be reproducibly implanted in less than 5 μl.

Second, these data demonstrate that genetically engineered HSV mutants can be used for the specific purpose of treatment of brain tumors without the requirement for alternative therapies (antiviral drugs) or the risk of progressive disease. While the usefulness of the $\gamma_1 34.5^-$ virus has been demonstrated in another model (13), we show that the virus in which the $\gamma_1 34.5$ gene is interrupted by a stop codon (R4009) rather than by deletion (R3616) appears to be more efficient in destroying tumor cells. We attribute this greater survival benefit to enhanced replication competence of R4009 as compared to R3616. One explanation of this observation is that a low level of stop codon suppression takes place and that the low level of expression of $\gamma_1 34.5$ enables the virus to effectively destroy tumor cells and yet not multiply to a level where it can cause encephalitis. The key to the development of effective oncolytic viruses may well depend on precise control of the expression of the $\gamma_1 34.5$ gene, and this observation may be exploited to construct still more effective viruses. Recently, other laboratories have assessed the value of alterations at other sites within the HSV genome for the creation of viruses suitable for treatment of brain tumors (28).

Human gliomas remain a devastating problem in human cancer, accounting for ≈5000 brain tumors yearly in the United States. Survival of patients 1 year after diagnosis remains <5%, even with existing chemotherapeutic modalities. Because of the profound and uniform morbidity, these tumors contribute more to the cost of cancer on a per capita basis than any other tumor. Development of viruses with increased selectivity for gliomas is in the forefront of potentially novel therapeutics for the treatment of this malignancy in hopes of improving long-term outcome.

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