Effects of Intravenously Administered Recombinant Vesicular Stomatitis Virus (VSVΔM51) on Multifocal and Invasive Gliomas

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Background: An ideal virus for the treatment of cancer should have effective delivery into multiple sites within the tumor, evade immune responses, produce rapid viral replication, spread within the tumor, and infect multiple tumors. Vesicular stomatitis virus (VSV) has been shown to be an effective oncolytic virus in a variety of tumor models, and mutations in the matrix (M) protein enhance VSV’s effectiveness in animal models. Methods: We evaluated the susceptibility of 14 glioma cell lines to infection and killing by mutant strain VSVΔM51, which contains a single–amino acid deletion in the M protein. We also examined the activity and safety of this strain against the U87 and U118 experimental models of human malignant glioma in nude mice and analyzed the distribution of the virus in the brains of U87 tumor–bearing mice using fluorescence labeling. Finally, we examined the effect of VSVΔM51 on 15 primary human gliomas cultured from surgical specimens. All statistical tests were two-sided. Results: All 14 glioma cell lines were susceptible to VSVΔM51 infection and killing. Intratumoral administration of VSVΔM51 produced marked regression of malignant gliomas in nude mice. When administered systemically, live VSVΔM51 virus, as compared with dead virus, statistically significantly prolonged survival of mice with unilateral U87 tumors (median survival: 113 versus 46 days, P = .0001) and bilateral U87 tumors (median survival: 73 versus 46 days, P = .0025). VSVΔM51 infected multifocal gliomas, invasive glioma cells that migrated beyond the main glioma, and all 15 primary human gliomas. There was no evidence of toxicity. Conclusions: Systemically delivered VSVΔM51 was an effective and safe oncolytic agent against laboratory models of multifocal and invasive malignant gliomas, the most challenging clinical manifestations of this disease. [J Natl Cancer Inst 2006;98:1546–57]

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resistant to conventional chemotherapies than their noninvasive counterparts (6). Therefore, malignant glioma must be considered as a cerebral disease that requires treatment not only of a single, main tumor mass but also of invasive cells and multiple tumor foci.

Recently, several oncolytic viruses have shown promising results in preclinical models of brain tumors. These viruses include reovirus (7–9), recombinant herpes simplex virus (10–14), Newcastle disease virus (15–17), recombinant poliovirus (18,19), myxoma virus (20), modified adenovirus (21–23), and wild-type vesicular stomatitis virus (VSV) (24–28). Despite impressive preclinical data, there are potential limitations to the use of these viruses in patients, such as inadequate distribution and/or delivery and insufficient levels of gene transfer or virus replication (29–32). Indeed, no dramatic results have been reported in the small number of early clinical trials in humans using oncolytic viruses against malignant glioma (15,16,33–36). Case reports of long-term survivors (35) and a patient with a complete response (17) have been described, and a clinical trial in patients with recurrent malignant gliomas treated with reovirus has been completed (37), but a final report has not been published.

Oncolytic viruses exploit a number of genetic defects in tumor cells (38–42). A common genetic defect occurring during tumor evolution is diminished responsiveness to interferon (43,44). This common defect reflects the important role of interferon-regulated pathways in the control of normal growth and apoptosis. Interferon is also a key mediator of the individual cell’s innate antiviral response. When tumor cells acquire mutations that allow them to escape interferon-mediated growth control pathways (e.g., those controlling proliferation or apoptosis), the tumor cells simultaneously compromise their innate viral responses, permitting a lethal viral infection within the tumor cell. In addition, tumor cells may have defects in signaling pathways such as the Myc, Ras, or p53 pathways that render them susceptible to VSV replication (26,28,45). Thus, tumor cells undergo growth and proliferation at the expense of losing their resistance to viral infection, and a lethal oncolytic infection occurs.

We and others have found that wild-type VSV is a potent oncolytic virus in a number of tumor cell types, including gliomas (24,28,43–45) but is lethal to animals that have not been treated with interferon (43). Hence, we used a VSV mutant called VSVΔM51. VSVΔM51 has a single amino acid deletion of methionine-51 (M51) of the matrix (M) protein. One of the functions of the M protein is to block the nuclear to cytoplasmic transport of interferon-beta mRNA, thereby circumventing the cellular interferon response. The deletion of methionine-51 from the M protein of VSVΔM51 abolishes this block and restores interferon-mediated responses in normal cells. This mutant theoretically has an improved therapeutic value (that is, safer but retaining the same efficacy) compared with wild-type VSV because it induces a marked interferon response in normal cells but retains its full oncolytic effect against tumor cells both in vitro and in vivo (43).

During the past several years of oncolytic virus development, it has become apparent that insufficient viral delivery can be a key limitation in the treatment of brain tumors (29,38,40,43). Direct inoculation of virus into a tumor may be advantageous in the treatment of localized tumor, but focal necrosis, tissue planes, and high intratumoral pressure will still limit viral distribution. Intravascular administration is an attractive alternative and may allow for multiple administrations over a long period of time. Although an intact blood–brain barrier will affect delivery to the normal brain, the blood–tumor barrier is somewhat permeable and may provide a potential advantage over direct intratumoral inoculation in delivery to multifocal tumors and invasive tumor cells. In addition, intravascular injection is simpler, less expensive, and less invasive clinically than direct local delivery. A number of preclinical studies have shown that virus treatments can be delivered to brain tumors via intracarotid delivery (46–50), but only two studies have shown intravenous delivery of an oncolytic virus for the treatment of brain tumors (51,52). The systemic/vascular delivery of VSV mutants has previously been shown to be effective in animal models of cancer, including those that had already widely metastasized (43,53) or were multifocal (54).

In this study, we investigated the effects of intravenous delivery of VSVΔM51 for the treatment of brain tumors. We carried out a detailed evaluation of the oncolytic properties of VSVΔM51 in vitro, in vivo, and ex vivo in human malignant glioma surgical specimens. We also compared the effects of VSVΔM51 to those of another oncolytic virus, reovirus. Reovirus has activity against experimental models of malignant glioma, but a small number of glioma cell lines are highly resistant to infection and killing by this virus (8,9).

Materials and Methods

Cell Lines

Fourteen human glioma cell lines (U87, U118, U251, U343, U373, U63, SF126, SF188, SNB19, UC12, UC13, UC14, RG2, and 9L) and murine NIH3T3 and L929 fibroblast cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HS68 human foreskin fibroblast cells were a gift from Karl Riabowol (University of Calgary, Canada). All cells were propagated in Dulbecco’s modified Eagle medium/F12 (DMEM/F12, Hybri-care; ATCC, Manassas, VA) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified, 5% CO₂ incubator. Each cell line was tested regularly for mycoplasma contamination.

Transfection of Glioma Cells With Red Fluorescent Protein

FuGene transfection reagent (Roche Diagnostic Co, Indianapolis, IN) and an expression plasmid containing red fluorescent protein (RFP) (Clontech, Palo Alto, CA) were used for transfection of a glioma cell line (U87) with RFP, as previously described (7). Briefly, FuGene transfection reagent and RFP:DNA vector were incubated together for 30 minutes at room temperature in serum-free media. The DNA mixture was applied to U87 cells for 4 hours at 37 °C in serum-free media. FBS was then added to a final concentration of 10%. Cells were grown at 37 °C and 5% CO₂, and the culture medium was changed daily. After 4 days, transfected cells were selected for G418 antibiotic resistance (400 μg/mL) and identified by fluorescent microscopy. RFP expression was found in more than 95% of cells as determined by fluorescence-activated cell sorting; this method confirmed the purity of the U87-RFP-expressing cells.

Viruses and Cell Infection

VSVΔM51 is derived from the Indiana serotype of VSV and is propagated in Vero cells (African green monkey kidney cells).
VSVΔM51 has a single amino acid deletion of methionine-51 of the M protein and contains an extra cistron that encodes green fluorescent protein (GFP) inserted between the G and L sequences. This recombinant genome was used to generate a replication-competent, GFP-expressing VSV clone (43). Dead virus was prepared by exposing live virus to ultraviolet (UV) irradiation for 1 hour. Reovirus serotype 3 (strain Dearing or T3D) was grown in L929 mouse fibroblast cells and purified as previously described (55); it was similarly UV inactivated to generate dead virus. Tumor or normal cells grown to 50%–60% confluence in 96-well plates were infected in 50 μL of serum-free medium and incubated for 1 hour at 4 °C for reovirus or 37 °C for VSVΔM51. Medium (150 μL) was then added to each well, and cells were returned to 37 °C at 5% CO2 for use in subsequent experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-2H-Tetrazolium Bromide Assay

Viability of tumor or normal cells infected as above with different doses (multiplicity of infection [MOI] = 0, 1, and 10) of VSVΔM51 or reovirus-T3D was measured 24 hours (VSVΔM51) and 72 hours (reovirus) after infection by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, as previously described (55). Briefly, cells were incubated with MTT (1 mg/mL) at 37 °C and 5% CO2 for 1 hour and lysed with dimethyl sulfoxide, and the absorbance was read with an ultra microplate reader (Bio-Tek Instruments, Inc, Burlington, VT).

Assays to Measure Cytopathic Effect of VSVΔM51 or Reovirus Infection

All glioma and normal cell lines were seeded at 5 × 10⁴ cells per well in six-well plates and incubated at 37 °C in 5% CO2 overnight. After infection with live or dead VSVΔM51 at an MOI of 1.0 or 10 for 24–72 hours, the cells were examined using a Zeiss inverted microscope (Axiovert 200M) mounted with a Carl Zeiss camera (AxioCam MRC, Carl Zeiss Inc, Thornwood, NY) to obtain both phase contrast and fluorescent images (using a fluorescein isothiocyanate filter to visualize virus-encoded GFP).

Western Blot to Detect Viral Proteins

All cell lines were seeded at 5 × 10⁴ cells per well in six-well plates and incubated at 37 °C in 5% CO2 overnight. Cells were then treated with either dead or live VSVΔM51 virus at an MOI of 1. Twenty-four hours after infection, the cells were collected by scraping and were lysed in 500 μL of lysis buffer (20 mM Tris pH 8.0, 136 mM NaCl, 10% glycerol, 1% NP40, 0.02% leupeptin, 0.5% aprotinin, and 1.5% sodium orthovanadate) for 20 minutes. Cellular debris was removed by low-speed centrifugation (1000g for 10 minutes) at 4 °C. Protein concentration of cell lysates was determined using the BCA protein assay kit (Biolynx, Inc, Brockville, ON, Canada). Supernatants were frozen at −80 °C for long-term storage. For electrophoresis, equal amounts of protein were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with polyclonal anti-VSV antibody (1:1000) overnight at 4 °C, washed three times with 1× Tris buffered saline, and incubated for 1 hour with horseradish peroxidase (HRP)–conjugated secondary antibody (1:3000 dilution, Biosciences, Amersham, Piscataway, NJ) at room temperature (John C. Bell, unpublished). Antibody binding was detected using an enhanced chemiluminescence reagent (Biosciences, Amersham) according to the manufacturer’s instructions.

Mice

CD-1 nude mice (female, 6–8 weeks old) were purchased from Charles River Canada, Constant, PQ, Canada. Three to four mice were caged together in each vivarium with a 12-hour light/dark schedule at 22 ± 1 °C and a relative humidity of 50 ± 5%. Food and water were available ad libitum. In all experiments, mice were killed by cervical dislocation when they had difficulty ambulating, feeding, or grooming or had lost at least 20% of their body weight. All animal procedures were reviewed and approved by the University of Calgary Animal Care Committee.

In Vivo Oncolyis in Subcutaneous Tumor Model

Female CD-1 nude mice (n = 21) were each implanted with 1 × 10⁶ U87 (n = 11) or U118 (n = 10) malignant glioma cells on the right flank to establish subcutaneous tumors. Tumor size (length × width) was measured daily using calipers. When palpable tumors reached approximately 25 mm², mice were treated intratumorally with live or dead VSVΔM51 virus (three injections of VSVΔM51 1 × 10⁶ plaque-forming units [PFU] at 2-day intervals) (8). Tumor size was then measured until sacrifice was indicated.

Determination of the Toxicity of the Intracerebral Administration of VSVΔM51 in Nude Mice

Female CD-1 nude mice (n = 8) were injected intracerebrally with either dead or live VSVΔM51 (5 × 10², 1 × 10³, 1 × 10⁴ PFU per mouse, two mice per dose) at a depth of 3 mm under guidance of a stereotactic frame (Kopf Instruments, Tujunga, CA), as described previously (7,20,56). Briefly, virus was injected intracerebrally into the right putamen. A 0.5-mm burr hole was made 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture through a scalp incision. Stereotactic injection used a 5-μL syringe (Hamilton Co, Reno, NV) with a 30-gauge needle, inserted through the Burr hole to a depth of 3 mm, mounted on a Kopf stereotactic apparatus (Kopf Instruments). After 10 seconds, the needle was withdrawn and the incision was sutured. Mice were followed daily for toxic effects. After the mice were killed, their brains, lungs, kidneys, hearts, and livers were removed, fixed in 10% formalin, embedded in paraffin, and sectioned with a microtome. Sections were stained with hematoxylin and eosin for histologic analysis, analyzed for VSV antigens by immunohistochemistry, or analyzed for the presence of DNA fragments by terminal transferase deoxyuridine triphosphate nick-end labeling (TUNEL) assay.

Immunohistochemistry to Detect VSV Antigens

Frozen sections of mouse brain and major organs (heart, liver, lung, and kidney) were fixed in 4% paraformaldehyde for 20 minutes and washed three times in phosphate-buffered saline (PBS). Sections were then exposed to primary polyclonal rabbit anti-VSV antibody at a 1:3000 dilution in PBS containing 2%...
bovine serum albumin, for 24 hours at 4 °C. Biotinylated anti-rabbit IgG (Vector Laboratories, Burlington, CA) was used as the secondary antibody. Sections were then incubated with avidin conjugated to HRP (Vectastain ABC immunohistochemistry kit, Vector Laboratories), and staining was visualized by addition of the DAB (3,3'-diaminobenzidine) substrate. To visualize VSV antigens, sections were mounted and viewed with a Zeiss inverted microscope (Axiovert 200M) and a Carl Zeiss camera (AxioCam MRc) to obtain both phase contrast and fluorescent images.

**TUNEL Assay**

The presence of fragmented DNA was analyzed with the TUNEL technique using the ApopTag plus fluorescein in situ apoptosis detection kit (Chemicon, Inc, Temecula, CA) according to the manufacturer’s instructions. Briefly, paraffin-embedded brain sections were dewaxed in xylene, rehydrated in an ethanol gradient, and treated with proteinase K (Invitrogen, Carlsbad, CA; 20 μg/mL in PBS) for 20 minutes at room temperature. Sections were washed in PBS, incubated with reaction mixture including terminal deoxynucleotidyl transferase and fluorescein-dUTP for 1 hour at 37 °C, washed in PBS, incubated with the antidigoxigenin conjugate for 30 minutes at 37 °C, and then counterstained with 4',6-diamidino-2-phenylindole.

**Sequencing of M Protein in VSV\textsuperscript{AM51}**

At 48 hours after intracerebral injections of 1 × 10^4 PFU of VSV\textsuperscript{AM51}, mice were killed by cervical dislocation and their brains were homogenized in PBS (pH 7.2). Virus was amplified by a single passage of brain homogenate on Vero cells for 24 hours. Virions were purified from the Vero cell supernatant by passage through a 0.2-μm filter followed by centrifugation at 30 000g for 90 minutes. The virus pellet was resuspended in PBS, and genomic RNA was extracted by sequential addition of the DAB, and genomic RNA was extracted by sequential addition of the DAB (3,3'-diaminobenzidine) substrate. To visualize VSV antigens, sections were mounted and viewed with a Zeiss inverted microscope (Axiovert 200M) and a Carl Zeiss camera (AxioCam MRc) to obtain both phase contrast and fluorescent images.

**Determination of the Appropriate VSV\textsuperscript{AM51} Dose for Intravenous Administration in Nude Mice**

Female CD-1 nude mice (n = 24) received intravenous injection of dead or live virus (at doses of 5 × 10⁷, 5 × 10⁸, 1 × 10⁹, or 5 × 10⁹ PFU per mouse) via the tail vein. Mice were followed for up to 60 days, and their body weights were recorded every other day. After the mice were killed by cervical dissection, their brains and major organs (liver, lung, heart, and kidney) were saved either for virus recovery assays in liquid nitrogen or pathologic analysis in formalin as described above. For the in vivo therapeutic experiments, we selected a dose that was one dose level below the dose at which 50% of the mice died; we refer to this dose as the maximum tolerated.

**Virus Recovery Assays**

Mice were killed by cervical dislocation, and saline was immediately infused into the left ventricle of the heart. Tissues were extracted and then homogenized in liquid nitrogen using a Pellet Pestles Kit (VWR International, Edmonton, Alberta, Canada) followed by repeated freeze–thawing to release virus from the cells. Supernatants were used for plaque titration on Vero cells as previously described (58). Briefly, Vero cells were plated in six-well plates and infected with serial dilutions of sample supernatant. Forty-eight hours after incubation at 37 °C in 5% CO₂, cells were overlaid with 2× MEM (Mediatech, Herndon, VA) and 2× Noble agar (Difco Laboratories, Detroit, MI) containing 0.2 mL neutral red (Sigma Chemical, Oakville, Ontario, Canada). Virus plaques were counted, and PFU were calculated by the number of plaques multiplied by the dilution factor.

**Survival Studies in an Orthotopic Human Glioma Model of Nude Mice**

To investigate the antitumor efficacy of VSV\textsuperscript{AM51} in mice, an orthotopic unilateral glioma animal model was established with the human glioma cell line U87. The stereotactic techniques used to implant glioma cells in the right putamen have been described previously (7,20). Briefly, female CD-1 nude mice (n = 13) were anesthetized, and U87 glioma cells (1 × 10⁵ cells per mouse) were implanted under the guidance of a stereotactic frame, as described above. After 15 days, mice were injected intravenously via the tail vein with multiple doses of live (n = 8) or dead (n = 5) virus (5 × 10⁸ PFU per mouse every 2 days, for a total of three injections). Mice were monitored every other day for survival. After the mice were killed, their brains and major organs were prepared as described above for histologic analysis, immunohistochemical analysis of VSV antigen, and TUNEL assay to assess apoptosis.

Survival was also assessed in mice with bilateral brain tumors. To prepare these mice, we implanted U87 cells (5 × 10⁵ cells per mouse per side in both sides of the brain) in CD-1 nude mice (n = 11). After 11 days, mice were injected intravenously, via the tail vein, with live (n = 6) or dead (n = 5) virus (5 × 10⁸ PFU per mouse every other day for three injections and every 5 days for another three injections for a total of six injections). Survival was followed, and organs were analyzed as above.

**VSV\textsuperscript{AM51} Viral Distribution Studies**

To determine if VSV\textsuperscript{AM51} targets multifocal gliomas in the brain, we established a dual tumor model using U87 tumor cells. Cells were implanted by stereotactic techniques as described above in CD-1 nude mice (n = 18). After 15 days, each mouse was injected intravenously, via the tail vein, with a single dose of VSV\textsuperscript{AM51} (5 × 10⁸ PFU per mouse). Mice were killed at the following time points after virus injection (three mice at each time...
Primary Human Glioma Culture

Short-term cultures were established from patient samples of human gliomas (n = 15) obtained following brain tumor surgery at the Foothills Hospital (Calgary); this study was approved by the Conjoint Medical Ethics Committee. Briefly, each patient specimen was split in two pieces; one portion of the specimen was fixed in 10% formalin and the other portion of the specimen was used for short-term cultures. The tumor tissue that was used to establish short-term cultures was washed several times in sterile saline, transferred to a 35-mm tissue culture dish, cut into small pieces (approximately 0.5–1 mm in diameter), and dissociated with trypsin (0.25%) and 50 μg/mL deoxyribonuclease (Roche Diagnostics, Laval, PQ, Canada) for 30 minutes at 37°C. After filtering and washing with DMEM/F12 (containing 20% FBS), cells were resuspended in 20% FBS in DMEM/F12 and plated at (10 000–100 000 cells per well) in 96-well plates. Cells were infected the following day with VSVΔM51 virus, both live and UV inactivated, at MOIs of 0.1, 1, and 10. Cell viability was measured 72 hours later by MTT and cytopathic effect assays [as above and in (20)].

Primary Tumor Immunocytochemistry for Glial Fibrillary Acid Protein Expression

Primary tumor cells obtained from surgical specimens were grown in eight-well chamber slides (50 000–200 000 cells per well) and fixed for 15 minutes in 4% paraformaldehyde. Cells were then blocked with 10% goat serum and 0.1% Triton X-100 in PBS for 30 minutes. Primary antibody (glial fibrillary acidic protein [GFAP] monoclonal antibody 1:1000, Chemicon, Inc) was then added. After 24 hours at 4°C, cells were washed with PBS and then incubated with anti-mouse IgG-Cy3 (Vector Laboratories) for 1 hour. Sections were mounted with Geltol mounting medium (Fisher scientific Co, Pittsburgh, PA) and viewed with a Zeiss microscope (Axiovert 200M), and pictures were taken with a Zeiss inverted microscope and a Carl Zeiss camera (Axiocam MRC).

Statistical Analyses

Statistical Analysis Software (SAS Institute, Inc, Cary, NC) and GraphPad Prism (version 4; GraphPad Software Inc, San Diego, CA) were used for statistical analyses. Survival curves were generated by the Kaplan–Meier method. The log-rank test and two-way analysis of variance (ANOVA) were used to compare the effect of different forms of treatment (live virus versus dead virus) and the time since administration on tumor size. All reported P values were two-sided and were considered to be statistically significant at P<0.05.

RESULTS

Susceptibility of Human Malignant Glioma Cell Lines to Infection and Killing by VSVΔM51

Fourteen malignant glioma cell lines were tested for susceptibility to infection and killing by VSVΔM51 and reovirus T3D by cytopathic effect and MTT assays (representative examples in Fig. 1). All 14 cell lines were susceptible to infection and killing by VSVΔM51, whereas only 12 (85%) of the lines were susceptible to infection and killing by reovirus (U118 and U343 were resistant). In contrast, neither of the normal cell lines tested (HS68 and NIH3T3) was killed by VSVΔM51 or reovirus. Cells were infected with either 1 MOI of VSVΔM51 or 10 MOI of reovirus; complete cell death was observed in all glioma cell lines 48 hours after infection with VSVΔM51 (Fig. 1, A) but not until 144 hours after infection with reovirus (Fig. 1, B). Untreated cells and cells treated with UV-inactivated (dead) virus did not exhibit any cytopathic effect at any time point assessed.

The MTT viability assay confirmed the results from the cytopathic effect assays (Fig. 1, C). VSVΔM51 produced more rapid glioma cell killing and at a lower (1 MOI, 24 hours) MOI than reovirus T3D (10 MOI, 72 hours) (Fig. 1, C). Extensive cell killing was found in all glioma cell lines in the presence of VSVΔM51. The normal cell lines NIH3T3 and HS68 were not susceptible to either reovirus or VSVΔM51 (Fig. 1, C). To ensure that cell killing following treatment with VSVΔM51 was due to viral infection rather than a nonspecific effect, viral protein production was assessed by western blotting. All glioma lines exhibited viral protein production 24 hours after infection, in accordance with their susceptibility to killing by VSV (Fig. 1, D), whereas no viral protein production was seen in normal cell lines (Fig. 1, D).

Effects of Intratumoral Administration of VSVΔM51 on a Glioma Cell Line Resistant to Infection by Reovirus

We next investigated if VSVΔM51 would infect and kill a cell line resistant to reovirus in a subcutaneous tumor in CD-1 nude mice. We treated mice bearing subcutaneous human malignant glioma xenografts of U87 (susceptible to reovirus) and of U118 (resistant to reovirus). These cells were implanted in the hind flanks of mice, which were treated intratumorally with live or dead virus after palpable tumors had formed (1 × 10⁷ PFU per mouse, every other day, for a period of 6 days). We observed statistically significant inhibition of tumor growth in the live virus–treated mice compared with the dead virus–treated control mice bearing either U87 (ANOVA, P<0.001) (Fig. 2, A) or U118 (two-way ANOVA, P<0.001) tumors (Fig. 2, B).
Toxicity of Intracerebrally Administered VSVΔM51 in CD-1 Nude Mice

We evaluated the toxicity of VSVΔM51 in vivo when administered intracerebrally. A single intracerebral administration of VSVΔM51 was lethal, even at a dose as low as $5 \times 10^2$ PFU per mouse (Fig. 3, A). This experiment was repeated three times with similar results (data not shown). Following intracerebral administration we found a diffuse meningoencephalitis, a marked tropism for neurons, and neuronal apoptosis. This was severe in the hippocampus (Fig. 3, B) and brain stem (data not shown), and the affected areas were positive for VSV antigen expression and TUNEL staining (Fig. 3, B).

We were surprised that the mutant VSVΔM51, which was engineered to enhance the antiviral responses in normal cells, retained substantial neurotoxicity when administered intracerebrally. To exclude the possibility that the VSVΔM51 had reverted to wild-type VSV (i.e., lost the mutation in the M protein), we sequenced the M protein of virus collected from mice after intracerebral administration. We confirmed that the virus cultured from the brains retained the mutation in the M protein and had not reverted to wild-type VSV.

Safety Evaluation of Intravenous Administration of VSVΔM51 in Nude Mice

We next evaluated the toxicity and maximum tolerated dose of VSVΔM51 administered intravenously. The mice tolerated much higher doses of intravenously than intracerebrally administered VSVΔM51. All mice (n = 4 per group) administered intravenously, via the tail vein, with doses of $\leq 1 \times 10^9$ PFU survived and appeared normal for up to 60 days (Fig. 4, A), when we terminated the experiment by killing all mice that were still alive. At a dose of $5 \times 10^9$ PFU (the highest dose we were technically capable of preparing), however, only 50% (two of four) of the mice survived for 60 days (Fig. 4, A). A transient and not statistically significant weight loss was observed 3–11 days after intravenous virus administration in surviving mice of both the $5 \times 10^9$ PFU and the $1 \times 10^9$ PFU groups (Fig. 4, B). Control mice treated with dead virus appeared normal. Hence, $1 \times 10^9$ PFU was the maximum tolerated dose when administered intravenously, and we used one dose level below this dose for all subsequent therapeutic experiments.

The histology of major organs (brain, liver, lung, kidney, and heart) of mice treated with a dose of $5 \times 10^8$ PFU was normal, and there was no histologic evidence of apoptosis by TUNEL assay (data not shown). In addition, we detected no viral antigen expression in liver, lung, kidney, or heart by immunohistochemistry, although in the brain there was minor VSV antigen staining within the meninges or ventricle at 24 and 72 hours after intravenous administration of virus (data not shown). Similar results were obtained by virus recovery assay. Virus was detected in the brain (but not in any other organs) only beginning 4 hours after intravenous administration, peaking at 24 hours, and declining thereafter (data not shown).
Survival Following Systemic Intravenous Administration of VSVΔM51 in CD-1 Nude U87 Tumor–Bearing Mice

We evaluated the effect of VSVΔM51 delivered intravenously to mice with unilateral or bilateral brain tumors. Unilateral or bilateral U87 glioma orthotopic xenograft models were established by intracerebral inoculation of U87 cells into the brains of CD-1 nude mice. Mice were treated intravenously with multiple doses of VSVΔM51 either 15 days (for unilateral tumors) or 11 days (for bilateral tumors) after tumor implantation. Mice with unilateral tumors treated with live virus survived statistically significantly longer (mean = 113 days, 95% CI = 96 to 130 days) than those treated with dead virus (mean = 46 days, 95% CI = 39 to 53 days, log-rank test, \( P = .0001 \)) (Fig. 5, A). All mice eventually died from recurrent tumors between 35 and 140 days after tumor implantation. Similar, although less dramatic, results were found in mice harboring bilateral U87 human malignant gliomas.

Histologic analysis showed that all live virus–treated mice died from a large tumor in the brain, with some live virus–treated mice having slightly larger ventricles than dead virus–treated mice (data not shown). There was no histologically evident change in the hippocampus region of the brain, immunohistochemical staining evidence of viral infection of the hippocampus, or evidence of apoptosis by TUNEL staining of the hippocampus (Fig. 5, C).

Infection of Multifocal Gliomas and Invasive Tumor Cells With Intravenous VSVΔM51

Having shown that intravenous VSVΔM51 prolonged survival of mice bearing gliomas, we evaluated whether a productive infection occurred in the tumors and characterized the distribution of infection in the tumor margin and invasive glioma cells. Mice (n = 18) were implanted in both brain hemispheres with U87 cells and were administered a single intravenous dose of \( 5 \times 10^8 \) PFU per mouse GFP-labeled VSVΔM51 15 days after tumor implantation. Mice were killed at multiple time points (three mice per time point), and their brains were examined in detail.
GFP-expressing virus, as visualized by fluorescence microscopy, was confined to the tumor, with no expression elsewhere in the normal brain, lung, kidney, liver, or heart. Viral expression of GFP began 10 hours after infection, increased up to 72 hours, decreased slightly by day 7, and was undetectable by 15 days after viral administration (Fig. 6, A). The viral titers from the tumor tissues, as determined by virus recovery assays (Fig. 6, B), confirmed that a productive viral infection occurred in these tissues and that the infection had a similar temporal profile to the results based on fluorescence microscopy. No evidence of replicating virus was found in non–tumor-containing brain tissue (data not shown) or dead virus–treated brains (data not shown).

To determine whether VSVΔM51 infects invasive glioma cells that have migrated beyond the main glioma mass, we inoculated U87 cells that had been transfected with an expression plasmid containing RFP into the brains of six CD-1 nude mice. Fifteen days after implantation, a single intravenous dose of VSVΔM51 was administered, and (based on the in vivo viral distribution results above) the mice were killed 72 hours later. We then examined the sections of the brain using fluorescence microscopy and immunohistochemistry. We found that viral GFP colocalized with the tumoral RFP using fluorescence microscopy and colocalization of VSV proteins and tumor cells by immunohistochemistry (Fig. 6, C). In addition, viral GFP expression colocalized with isolated invasive tumor cells at the margins of the tumor (Fig. 6, C).

Fig. 5. Systemic administration of vesicular stomatitis virus strain AM51 (VSVΔAM51) and survival of nude mice with unilateral and bilateral U87 cell–derived brain tumors. A) Unilateral tumor model. Kaplan–Meier survival analysis of mice implanted with U87 cells (1 × 10^5 cells per mouse) and treated with either dead (n = 5) or live (n = 8) virus every 2 days for a total of three doses. One intravenous injection contained 5 × 10^7 plaque-forming units (PFU) per mouse. All P values are two-sided. B) Bilateral tumor model. Kaplan–Meier survival analysis of mice implanted with U87 cells (5 × 10^4 cells per liver per side) and injected with either dead (n = 5) or live (n = 6) virus (5 × 10^8 PFU per mouse) three times every 2 days followed by three times every 5 days for a total of six injections. All P values are two-sided. Arrows in (A) and (B) indicate virus injections. C) Representative photomicrographs of a whole brain slice from a live virus–treated mouse (magnification: left, ×25; right, ×400). Top row of panels shows hematoxylin and eosin staining (H&E) of the hippocampus region, middle row of panels shows immunohistochemical (IHC) staining of the VSV antigen in the hippocampus region, and bottom row of panels shows terminal transferase deoxyuridine triphosphate nick-end labeling (TUNEL) assay of the hippocampus. Adjacent brain sections were used. Arrows indicate the tumor.

Fig. 4. Safety and toxicity evaluation of vesicular stomatitis virus strain AM51 (VSVΔAM51) when administered intravenously to nude mice. A) Intravenous administration of VSVΔAM51 in CD-1 nude mice at doses of 5 × 10^7, 5 × 10^8, 1 × 10^9, or 5 × 10^9 plaque-forming units (PFU) per mouse (n = 4 per group). Mice were given a single injection of dead or live VSVΔAM51 intravenously, via the tail vein, and monitored for 60 days. B) Mean body weight over time of mice intravenously administered VSVΔAM51.
Infection and Killing by VSVΔM51 of Primary Human Malignant Gliomas Cultured From Surgical Specimens

We next determined whether the in vitro cell line results would also apply to glioma samples from patients. Accordingly, we tested whether VSVΔM51 could infect and kill short-term glioma cultures derived from glioma surgical specimens. We examined the susceptibility to VSVΔM51 of 15 ex vivo brain tumor surgical specimens derived from four glioblastomas, five oligodendrogliomas, five astrocytomas, and one gliosarcoma. All specimens tested (15/15) were killed by VSVΔM51 infection (Fig. 7, A) and to a degree that was similar to infection and killing of U87. A widespread cytopathic effect and viral GFP expression was seen in live virus–treated glioma cells (Fig. 7, B-ii, iii, v, vi). In contrast, short-term cultures treated with dead virus showed no cytopathic effect or viral GFP expression (Fig. 7, B-i, iv). GFAP staining confirmed the glial lineage of the astrocytic specimens (e.g., a glioblastoma) (Fig. 7, C-ii), which was also apparent morphologically using hematoxylin and eosin staining (Fig. 7, C-ii, iii).
DISCUSSION

We found that an attenuated VSV mutant, designated VSV\(^{\Delta M51}\), infected and killed all malignant glioma cell lines tested and did not infect normal cells in vitro. When administered intravenously to CD-1 nude mice bearing human gliomas, VSV\(^{\Delta M51}\) dramatically prolonged their survival and infected both multifocal gliomas and invasive glioma cells. The invasive and multifocal nature of glioma are major clinical challenges in treating this disease.

An ideal oncolytic virus for cancer should have several characteristics \((38,42,59)\). It should have effective delivery into multiple sites within the tumor, evade innate and acquired immune responses, produce rapid viral replication, spread within the tumor, and infect multifocal tumors. It should also be "engineerable" so that it can be modified to, for example, improve its efficacy or tumor targeting. This is precisely what we found using the attenuated live virus we constructed (VSV\(^{\Delta M51}\)). Its efficacy was maintained and the antiviral responses were enhanced in normal cells, thereby improving its therapeutic index \((43)\). In addition, we found that VSV\(^{\Delta M51}\) infects both multifocal gliomas and invasive glioma cells. Conventional therapies such as surgery and radiotherapy are ineffective in treating invasive glioma cells because these cells may have become more resistant to these therapies than the tumor cells in the main tumor mass by adopting a number of cellular characteristics (e.g., increasing expression of survival pathways, decreasing expression of apoptotic programs, reduced proliferation, etc.) \((60)\). Hence, VSV\(^{\Delta M51}\) may represent an effective treatment for these chemotherapy-resistant tumor cells.

We compared the effectiveness and toxicities of VSV\(^{\Delta M51}\) to reovirus in this study on the basis of our previous work \((8,9,55)\). We found that VSV\(^{\Delta M51}\) was superior in several ways to reovirus for treating gliomas. VSV\(^{\Delta M51}\) killed all glioma lines we tested (including two that were resistant to reovirus), was effective in vivo when administered systemically, infected invasive glioma cells, and killed multifocal gliomas. By contrast, reovirus did not cause regression of bilateral gliomas in an immunocompetent racine glioma model \((55)\). However, reovirus is superior to VSV\(^{\Delta M51}\) in many other ways. When administered intratumorally, reovirus "cures" experimental gliomas in the majority of glioma-bearing mice (i.e., mice survived until the experiment was arbitrarily terminated 90 days after tumor implantation, often without histologic evidence of residual tumor) \((8)\), whereas we found that intravenous VSV\(^{\Delta M51}\) did not cure any mice. Finally, unlike VSV\(^{\Delta M51}\), reovirus is benign when administered intracerebrally in adult nude mice \((8)\) or immunocompetent rats \((55)\). Comparisons between oncolytic viruses of putative efficacy or toxicities in animal models at present are very limited \((61)\). Definitive conclusions regarding efficacy or toxicity await the testing of these oncolytic viruses in clinical trials in malignant glioma patients.

Our study has several limitations. First, we have not yet evaluated VSV\(^{\Delta M51}\) in immunocompetent models of gliomas. Such evaluation is important because immune responses (partially ablated in the immunocompromised mice we used here) may limit delivery of the virus to the tumor when administered intravenously. Immune responses may also limit the distribution of the virus within the tumor. Second, the precise mechanism by which intravenous VSV\(^{\Delta M51}\) accesses the invasive glioma cells, which in many cases appear to be single cells, is unknown. The main tumor mass of U87 is highly vascularized and has a "leaky" blood–brain barrier, which would allow intravenously delivered virus access to the main tumor mass \((62,63)\). In contrast, invasive glioma cells are not believed to be extensively vascularized \((64)\) and would therefore have limited contact with systemically delivered virus. We assume that higher pressure within the tumor mass \((65)\) and high concentrations of virus within the tumor, under pressure, may move the virus out along tracts of white matter. Because these invasive cells may be in a hypoxic environment, it should be noted that VSV is able to infect and kill hypoxic glioma cells both in vitro and in vivo \((24)\). Alternatively, peritumoral increases in neovascularity and tissue edema can increase the permeability of the blood–brain barrier \((66,67)\), allowing the virus to leak out into the perivascular interstitium around the vessels. Third, all the glioma-bearing mice ultimately died from recurrent tumor. We are now exploring strategies to understand the causes of treatment failure with the goal of improving its efficacy (e.g., using live imaging of viral delivery, improving blood–brain barrier breakdown and intra-arterial delivery, etc.).

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NOTES

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