Efficacy and Safety Evaluation of Human Reovirus Type 3 in Immunocompetent Animals: Racine and Nonhuman Primates

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ABSTRACT

Purpose: Human reovirus type 3 has been proposed to kill cancer cells with an activated Ras signaling pathway. The purpose of this study was to investigate the efficacy of reovirus in immunocompetent glioma animal models and safety/toxicity in immunocompetent animals, including nonhuman primates.

Experimental Design: Racine glioma cells 9L and RG2 were implanted s.c. or intracranially in Fisher 344 rats with or without reovirus antibodies, followed by treatment of reovirus. To study whether reovirus kills contralateral tumors in the brain and to determine viral distribution, we established an in situ dual tumor model followed by reovirus intratumoral inoculation only into the ipsilateral tumor. To evaluate neurotoxicity/safety of reovirus, Cynomolgus monkeys and immunocompetent rats were given intracranially with reovirus, and pathological examination and/or behavioral studies were done. Viral shedding and clinical biochemistry were systematically studied in monkeys.

Results: Intratumorally given reovirus significantly suppressed the growth of both s.c. and intracranially tumors and significantly prolonged survival. The presence of reovirus-neutralizing antibodies did not abort the reovirus’ antitumor effect. Reovirus inhibited glioma growth intracranially in the ipsilateral but not the contralateral tumors; viral load in ipsilateral tumors was 15 to 330-fold higher than the contralateral tumors. No encephalitis or behavioral abnormalities were found in monkeys and rats given reovirus intracranially. No treatment-related clinical biochemistry changes or diffuse histopathological abnormality were found in monkeys inoculated intracranially with Good Manufacturing Practice prepared reovirus. Microscopic changes were confined to the region of viral inoculation and were dose related, suggesting reovirus intracranially was well tolerated in nonhuman primates.

Conclusions: These data show the efficacy and safety of reovirus when it is used in the treatment of gliomas in immunocompetent hosts. Inoculation of reovirus into the brain of nonhuman primates did not produce significant toxicities.

INTRODUCTION

A number of replication competent oncolytic viruses have been selected or engineered as novel therapeutics for cancer (1–3). These viruses selectively infect and kill tumor cells but leave normal cells unaffected (or relatively so) by exploiting genetic defects that are unique to tumor cells. For several oncolytic viruses (e.g., reovirus, herpes simplex virus, vesicular stomatitis virus, and so on), it appears that tumor cells have defects in a number of antiviral pathways (e.g., double-stranded RNA-activated protein kinase and IFN) that render them susceptible to viral infection. Other viruses (e.g., herpes simplex virus, New- caster disease virus, measles, and adenovirus), including reovirus, are being tested in clinical trials.

We have previously discovered that reovirus (respiratory enteric orphan), a double-stranded RNA virus commonly isolated from the respiratory and gastrointestinal tracts of humans, infects, and lyses tumors cells but not normal cells (4–13). Reovirus does not cause disease in humans but can produce a lethal infection in neonatal (14) and SCID NOD (15) mice.
Reovirus usurps the activated ras signaling pathways of host tumor cells and targets malignant tumor cells with activated ras (4, 16–18). The restriction of reovirus replication in untransformed cells is due to the activation of the double-stranded RNA-activated protein kinase by early viral transcripts, which inhibits the translation of viral proteins (18). Activated ras or an activated element of the ras pathway inhibits (or reverses) RNA-activated protein kinase activation and allows viral protein synthesis and a lytic infection to occur. We have previously shown promising efficacy in several immunocompromised animal models of brain tumors (4, 5, 12, 13) but have neither evaluated its efficacy/toxicity in immunocompetent models of malignant gliomas nor its toxicity in nonhuman primates. Because it is likely that an intact immune response would affect the efficacy or toxicity of oncolytic viral therapy, we evaluated reovirus in immunocompetent hosts.

The objectives of this study were to determine the following: (a) the efficacy/toxicity of reovirus as an experimental therapeutic agent against malignant gliomas in immunocompetent model systems; and (b) the safety of the intracerebral inoculation of reovirus in immunocompetent hosts, including nonhuman primates. We found that reovirus prolongs survival in immunocompetent glialoma animal models, although its effect was less dramatic than in immunocompromised animals. The intracerebral administration of reovirus in Cynomolgus monkeys was safe and well tolerated.

MATERIALS AND METHODS

Cell Lines and Virus. The established glioblastoma cell lines U87, RG2, 9L, and U118 were obtained from the American Type Culture Collection (Manassas, VA). Cells were propagated in DMEM:Ham’s F-12 containing 10% fetal bovine serum. U87, RG2, 9L, and U118 were obtained from the American Type Culture Collection (Manassas, VA). Cells were propagated in DMEM:Ham’s F-12 containing 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. The cells were passaged when they reached ~80% confluence, harvested by trypsin treatment, and replated in DMEM:Ham’s F-12 containing 10% fetal bovine serum. Each cell line was tested routinely for Mycoplasma contamination.

Reovirus serotype 3 (strain Dearing) was grown and purified as previously described (7, 10), except that mercaptoethanol was omitted from the extraction buffer. Reovirus was propagated in suspension cultures of L929 cells and purified. Dead virus was prepared by exposing live virus to UV light for 45 minutes. Clinical-grade reovirus was used for primate studies, which was produced under Good Manufacture Practice guidelines under contract by BioReliance Corp. (Rockville, MD).

Transfection of GBM Cells with Green Fluorescence Protein (GFP). RG2 and 9L cells were transfected with GFP with FuGene transfection reagent (Roche Diagnostic Co., Indianapolis, IN), as described previously (12, 13). The G418 antibiotic (300 µg/mL for RG2 and 800 µg/mL for 9L) was used for transfection cell selection. GFP expression was >95% as determined by fluorescence-activated cell sorting.

Immuno­fluorescence Staining of Reovirus Infection. Infection of glioma cells with reovirus and immunofluorescence were done as described previously (12, 13).

Neutralizing Reovirus Antibody Determination. Rat serum was collected via tail vein and complement was inactivated at 56°C for 30 minutes before assay. Serum was serially diluted (up to 1:5120) with PBS, and 50 µL of the diluent were added in duplicate into each well of a 96-well plate. Fifty microliters of reovirus-3 [multiplicity of infection = 103 plaque-forming units (PFU)/mL] were added into diluted serum and the mixture incubated at 37°C for 2 hours. One hundred µL of L929 cell suspension (1 × 105 cell per mL) in serum-free medium were added to the serum/virus mixture, and the culture was incubated at 37°C in a humidified 5%CO2 atmosphere for 5 days. PBS and serially diluted L929 cell suspension were used as controls. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazonium bromide assay was then done to determine the viability of cells (after being lysed by residual or intact reovirus), as we described previously (12). The absorbance was read at 600 nm on an ELX-808 Ultra Microplate Reader (Bio-Tek Instruments, Inc.). Serum dilutions ≥ 1:10 were considered as antibody negative.

Viral Culture. Tissue samples underwent a freeze-thaw protocol and then were homogenized with an RNase-free Pellet Pestles Kit (VWR International, Edmonton, Alberta, Canada) or homogenizer (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany). Supernatants were clarified by centrifugation and diluted, and reovirus titers were determined with plaque assay on L929 cells as described previously (7). Clear body fluids were diluted and directly applied to L929 cells for virus titration.

Reverse Transcription-PCR (RT-PCR). Total RNA was extracted from body fluids or other samples with QIAamp Viral RNA Mini kit (Qiagen, Mississauga, Ontario, Canada). An equal amount of RNA was transcribed with random hexanucleotide primers, and PCR was done with reovirus s3 cDNA targeted primers: forward, 5′-GGGCTGCACATTACCACTGTA-3′; and reverse, 5′-CTCCTCGCAAATACAACCTCTG-3′. PCR reaction mixture was denatured at 95°C for 3 minutes and cycled for 35 cycles (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds) and then followed by extension at 72°C for 7 minutes. A 290-bp PCR product was expected and analyzed on a 1.5% agarose gel.

In situ Hybridization. Reovirus oligodeoxynucleotide of reovirus s1 gene was generated with RT-PCR with a forward primer 5′-AATTGATTAGTTAGTGACACTATAGCTATTGG-3′ and reverse primer 5′-TTAGTCGATGCATCATCGAGTCCC-3′. The purified product was cloned into pCR II vector with TOPO TA cloning kit (Invitrogen-Life Technologies, Inc., Burlington, Ontario, Canada), and the insert was verified with sequencing. Antisense and sense riboprobes were generated from the template (linearized with NotI) with SP6 RNA polymerase and T7 polymerase (linearized with SpoI, respectively). Probes were then labeled with dUTP according to manufacturers protocol (Roche Diagnostics, Indianapolis, IN). Briefly, frozen sections were air-dried, fixed in 4% paraformaldehyde, digested with proteinase K (20 µg/mL), acetylated, and then prehybridized in a buffer [containing 50% formamide, 5X saline-sodium phosphate-EDTA (3 mol/L NaCl, 173 mmol/L NaH2PO4, 25 mmol/L EDTA), and 1X Denhart’s solution (0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone)] for 4 to 6 hours at 55°C. Slides were hybridized at 55°C with 10 ng/mL probe and 8 µg/mL of Escherichia coli RNAIII. Sections were washed once in 2× SSC at 37°C and treated with 40 µg/mL RNase A at 37°C before going through sequential washes (2× SSC at 37°C, 2× SSC at 37°C, 2× SSC at 37°C, and 0.1× SSC at 37°C).
SSC at room temperature, 1× SSC at room temperature, 0.5× SSC at room temperature, and in 0.1× SSC at 50°C. Endogenous peroxidase was inactivated with a mixture of hydrogen peroxide/methanol. After blocking (PBS containing 10% horse serum), slides were incubated with digoxigenin monoclonal antibody (Boehringer Mannheim) overnight at 4°C and signals were detected with an avidin-biotin peroxidase complex kit (Vector Laboratories Inc., Burlington, Canada). Peroxidase was visualized with 3,3′-diaminobenzidine substrate and hematoxylin was used as a nuclear counterstain.

Western Blot Analysis. Tumors were smashed with a homogenizer (Ultra-Turrax T25, Janke & Kunkel) and went through freeze-thaw for three times and followed by centrifuging at 8000 rpm at 4°C. Supernatants were collected, and protein concentrations were measured by BCA protein assay. Protein samples were separated by SDS-PAGE, followed by electroblotting onto nitrocellular membrane. A polyclonal reovirus antibody (12) and horseradish peroxidase-conjugated secondary antibody were used to detect reovirus within tumor homogenate.

Animals. Eight- to 10-week-old female Fischer-344 rats (body weight 180 to 200 g; Harlan Sprague Dawley, Indianapolis, IN) were used in this study. The animals were housed in groups of three to four in a vivarium maintained on a 12-hour light/dark schedule with a temperature of 22 ± 1°C and a relative humidity of 50 ± 5%. Two- to 3-year-old Cynomolgus (Macaca fascicularis) monkeys (five male and five female; body weight 2.7 to 3.5 kg for the males and 2.9 to 4.3 kg for the females) were purchased from Health Canada Corporate Service Branch (Ottawa, Ontario, Canada). The monkeys were housed individually in stainless steel cages in an independent laboratory facility. The environment was maintained on a 12-hour light/darkness schedule with a temperature of 21 ± 3°C and a relative humidity of 50 ± 20%. Monkeys were acclimatized 11 weeks (including 10 weeks of quarantine). During the acclimation period, they were examined in detail by a clinical veterinarian, and blood and stool were collected for routine studies. Each animal was tested for tuberculosis, anti-helminitic therapy was given, and two immunizations for hepatitis A given. Animals were supplied with food and water ad libitum. All protocols were reviewed and approved by the Animal Care Committee of the independent laboratory and the University of Calgary. All animal work procedures were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals issued by NIH.

In vivo Studies with a Rodent s.c. Glioma Model in an Immunocompetent Host. Actively growing RG2 cells were harvested and resuspended in sterile PBS. Six- to 8-week-old Fischer 344 rats received injections of 10⁶ cells in a 100-μL volume in the hind flank. Tumors were allowed to grow until they measured 0.5 × 0.5 cm; animals were then given a series of intratumoral injections of either dead or live reovirus (5 × 10⁶ PFU of either RG2 or 9L cells suspended in 2 to 5 μL of PBS were injected subcutaneously at the tumor site, repeated 3 or 4 times at 4-day intervals. For experiments to assess tumor size, animals were sacrificed 18 days (an arbitrary time point) after tumor implantation. Brains were removed, processed, and prepared for H&E staining. Brain tumor sizes were obtained by using ImagePro software (Media Cybernetics, Silver Spring, MD). For experiments assessing survival, animals were followed until they lost at least 20% of body weight or had trouble ambulating, feeding, or grooming or until 160 to 220 days when they arbitrarily terminated the experiment. For experiments assessing effects of immunosuppression on efficacy of reovirus, tumor-bearing animals were also i.p. given 10 mg/kg/day cyclosporine (Sandimmune; Novartis Pharmaceu-
ticals Canada, Inc., Dorval, Quebec, Canada) started on day 4 (i.e., the same day of viral administration) and finished on day 100 of tumor implantation. For the experiments studying the effect of circulating neutralizing antibodies on the efficacy of reovirus, animals were immunized with 10^9 PFU of reovirus i.p. Two weeks later, when neutralizing antibodies to reovirus were detected in the serum, the animals were used for tumor allograft implantation and therapeutic purposes. At termination, animals were anesthetized, perfused intracardially with PBS, and then fixed by 4% paraformaldehyde. All of the brains and major organs of these animals were examined histologically.

**In vivo Viral Distribution Studies in an Orthotopic Glioma Model.** To examine viral distribution within the brain and to test the possibility of reovirus inhibiting remote glioma foci in situ, we intracranially implanted animals with GFP-transfected RG2 cells in both hemispheres and treated only one tumor (i.e., the ipsilateral tumor) with reovirus by direct intratumoral administration. Stereotactic inoculation was same as described above, except 5 × 10^4 of RG2-GFP cells were symmetrically implanted into each frontal lobe (2.5 to 3 mm posterior of coronal suture and 2.5 mm right or left of sagittal suture). Six days after tumor implantation when tumors were ~2 to 3 mm in diameter, an intratumoral injection of 1 × 10^6 PFU of either dead or live reovirus in 2 to 5 μL of PBS was given stereotactically only into the ipsilateral tumor over 20 seconds. Animals were sacrificed 2, 24, 48, 72, 96, and 144 hours after virus administration. When sacrificed, animals were anesthetized and perfused with 50 mL of saline, followed by 50 mL of phosphate-buffered 10% formalin via cardiac catheter. When dissecting, brain/tumor tissues were distinguished under a whole body fluorescent imaging system (12, 19). Both GFP-emitting tumors were saved frozen for viral culture. Other brains were dissected, weighed and examined histologically. Tissues were fixed by 4% paraformaldehyde. All of the brains and major organs of these animals were examined histologically.

**The Morris Water Maze.** Fischer 344 rats were inoculated intracranially with a therapeutically effective dose (10^9 PFU/rat, three times, 4-day interval) of live virus or dead virus or were untreated and followed for 120 days after infection. The animals were then evaluated with the Morris water maze. Healthy nonoperated Fischer 344 rats were used as normal controls. The Morris water maze was done as previously described (20–23) with minor modifications. Briefly, a large, round, white tank (150 cm in diameter and 45 cm in height) was filled with water (22°C to 23°C) to a depth of 26 cm. One hundred sixty to 180 g of milk powder were added into the tank to render the water opaque. The tank was surrounded by colorful shower curtains, which are thought to provide visual cues for the swimming rat. A square white platform (12 × 12 cm) was submerged 1 cm below the water level and placed in one of the quadrants. Rats were then released into the tank from four randomly selected positions and allowed to swim around the tank (for <90 seconds) until they encountered the platform. Each rat was allowed four trials (referred to as a trial block) every day for 6 days. Animals were allowed to stay on the platform for 10 s after each trial. Animals were then removed from the tank. The time to find the platform (escape latency; in seconds), and descriptions of the path followed in the tank were recorded and analyzed. As an additional control, on day 6, the platform was moved to the diagonally opposite quadrant and another trial block (probe trial block) was done. After the last trial, animals were anesthetized and perfused with 50 mL of saline, followed by 50 mL of phosphate-buffered 10% formalin via cardiac catheter. All brains and major organs were then prepared for conventional histopathological analysis.

**Intracerebral Inoculation of Reovirus into Nonhuman Primates.** Animals underwent an overnight fast before surgery. Ketamine hydrochloride was injected i.p. to induce anesthesia. The animals were given an i.v. bolus of Propofol, followed by a continuous Propofol infusion to maintain satisfactory anesthesia. Each monkey’s head was shaved, and a stereotactic head frame was placed. A midline incision was made, and the skull was exposed. A burr hole (2.5 mm in diameter) was made 2 mm anterior to the coronal suture and 7 mm left of the midline into the left frontal skull by using a Dremel drill, as described previously (24, 25). A Hamilton syringe mounted with a 25-gauge blunt-tipped needle was used to deliver the inoculate containing the virus. Four escalating doses (1.51 × 10^9, 1.51 × 10^7, 1.51 × 10^5, and 1.51 × 10^3 PFU) of good laboratory practice-quality reovirus (supplied by BioReliance Corporation, Rockville, MD) or 0.9% NaCl (control) were given to male and female monkeys (i.e., one male and one female received each dose level or control). The needle was lowered 6 mm from the surface of the brain through the burr-hole, and 100 μL of reovirus or sterile saline were injected into the cortex and white matter over 10 minutes. After injection, the burr hole was covered with bone wax and the incision sutured. After treatment, the monkeys were followed closely for a total of 2 to 6 months. Females were sacrificed at 2 months to determine short-term toxicity and males at 6 months to determine longer term toxicity. During follow-up, animals were assessed twice daily for vital signs, a detailed clinical examination done once a week and rectal body temperature recorded at least once daily. Clinical laboratory examinations (hematology, clinical chemistry, urinalysis, and blood coagulation testing) were done in all monkeys 1 day before and 17 days after treatment. Cerebrospinal fluid (collected on day 10 and before necropsy via cisternal puncture), blood (collected on days 1 and 15 and before necropsy), urine, stool, and nasal swabs (collected on days 2 and 15 and before necropsy) were collected for viral shedding studies via RT-PCR, rapid viral culture, and reovirus neutralization assay.

Upon the completion of the experiment, the animals were euthanized with sodium pentobarbital administered intravenously. All major organs (brain, adrenals, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus and uterus) were dissected, weighed and examined histologically. Tissues from a variety of organs (i.e., brain, cranium, pituitary, eyes, thyroid gland, salivary gland, thymus, trachea, heart, liver, stomach, gallbladder, small intestine, pancreas, colon, spleen, kidneys, testes and prostate gland or ovaries and uterus, bladder, skeletal muscle, skin, mammary gland, optic nerves, lymph nodes, femur & marrow and cervical spinal cord, etc.) were also prepared for histopathological examination. All tissues were examined by an independent certified veterinary pathologist and the brains examined in detail by a board-certified neuropathologist (C. Palmer). The animal protocols were approved by the
Animal Care Committee of the independent laboratory and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals issued by the NIH.

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 and Mann-Whitney tests were used to compare the distributions of survival times and tumor sizes, respectively. The average latency data were analyzed with the ANOVA two-way test. All reported P values were two-sided and were considered to be statistically significant at P < 0.05.

RESULTS

Susceptibility of Glioma Cells to Reovirus Infection In vitro. We characterized the susceptibility of two racine glioma cell lines 9L and RG2 to reovirus by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoium bromide assay, cytopathic effect, immunofluorescence, and [35S]methionine labeling to measure viral protein synthesis. The highly susceptible human glioma line U87 and resistant line U118 were used as positive and negative controls, respectively (5). We found these two racine cell lines were very susceptible to reovirus infection, with >50% (9L) or >90% (RG2) of cell killing within 72 hours of reovirus infection (data not shown). In contrast, cells receiving either dead virus, or no virus remained healthy. Immunofluorescence was done and indicated that cell killing resulted from reovirus infection (Fig. 1). To additionally confirm replication of reovirus in susceptible lines, we labeled cells with [35S]methionine before viral infection. Cell lysates were immunoprecipitated with a polyclonal reovirus antibody and analyzed by SDS-PAGE. A substantial amount of reovirus protein was found in U87, RG2, and 9L but not U118 cells 24 hours after reovirus infection (data not shown).

Intratumoral Inoculation of Reovirus Caused Regression of Gliomas Grown s.c. in Fischer 344 Rats. We first tested the effectiveness of reovirus against glioma in animals with a functional immune system by using a s.c. racine glioma model. Fischer 344 rats harboring established s.c. R2 tumors (~5 mm in diameter) were given a series of intratumoral injections of either dead virus or live virus (5 × 10^9 PFU/rat/day for the first week followed by 10^9 PFU/rat every 2 days for the next 3 weeks). On day 36 posttreatment, when the experiment was terminated because of heavy tumor burdens in dead virus-treated animals, the reovirus-treated animals had a significantly smaller tumor volume compared with control rats (two-way ANOVA, P = 0.0031; Fig. 2A). To test if reovirus causes glioma regression at a site distant from the site of virus administration in immunocompetent animals, we established bilateral RG2 tumors in both flanks of Fischer 344 rats followed by treatment of only the ipsilateral tumor with reovirus. The results showed that tumor growth inhibition or tumor regression was seen only in the live virus-treated ipsilateral (two-way ANOVA, P = 0.0064) but not contralateral tumors (two-way ANOVA P > 0.05; Fig. 2B). Substantial amounts of reovirus were detected in live virus-treated ipsilateral tumors, as measured by viral titers (Fig. 2C) and Western blot analysis (Fig. 2D) but not in contralateral (Fig. 2, C and D) or dead virus-treated tumors (data not shown). Because we could not reliably grow 9L tumors in the s.c. compartment, we were unable to do these experiments with s.c. 9L tumors.

Determination of the Maximum-tolerated Dose of Intracerebrally Administered Reovirus in Fischer 344 Rats. We observed that the maximum-tolerated dose of reovirus intracranially in nude mices is 10^8 PFU.12 Although reovirus is not thought to cause any known disease in adult immunocompetent rats.
animals [even when given intracranially (26)], we determined
the maximum-tolerated dose in Fischer 344 rats before carrying
any therapeutic experiments. All animals treated intracranially
with three administrations of $10^9$ PFU of reovirus or less re-
mained healthy and maintained or gained body weight (com-
pared with nonoperated or dead virus-treated groups: two-way
ANOVA, $P < 0.05$). Animals treated more often (e.g., five
times) or with higher doses (e.g., $4 \times 10^9$ PFU; the highest dose
we were technically capable of preparing) died or had a transient
loss of body weight (15 to 25%) 2 to 4 weeks after viral
inoculation (compared with control groups, two-way ANOVA
test, $P < 0.05$ or $P < 0.01$; data not shown). All nonoperated
or dead virus-treated rats remained healthy. Therefore, we selected
the highest dose (i.e., $1 \times 10^9$ PFU, three administrations) that
was well tolerated to be used in subsequent therapeutic exper-
iments.

Histologic examinations for the maximum-tolerated dose ex-
periments showed the following pathological findings: lethal dose
($4 \times 10^9$ PFU, five administrations, 4-day interval); severe inflam-
mation (lymphocytes, microglia, and macrophage infiltration)
found in subependymal areas and also in the hippocampus; and occasional necrosis or hemorrhage in the corpus callosum and inflammation that involved the parenchyma and subarachnoid space. These findings were consistent with severe diffuse meningoencephalitis. In addition, histologic examinations showed a moderate dose (2 \times 10^9 PFU, five administrations, 1 \times 10^9 PFU, five administrations, 4-day interval): prominent subependymal inflammation, inflammation in the subarachnoid space, localized calcification and dilated ventricles consistent with communicating hydrocephalus. In addition, histologic examinations showed a low dose (1 \times 10^9 PFU, three administrations, 4-day interval): subependymal lymphocytic infiltration, calcified lesions, focal inflammation, and dilated ventricles, the later consistent with communicating hydrocephalus.

**Intratumoral Administration of Reovirus Significantly Inhibited Tumor Growth and Prolonged Survival of Rats Bearing Intracerebral Gliomas.** To investigate the antitumor efficacy of reovirus in the brain in an immunocompetent racine model, 9L or RG2 glioma cells were stereotactically implanted into the putamen. Four days after tumor cell inoculation, when microscopic tumors had become established, the tumors were inoculated with single or multiple doses of live virus or dead virus. Results showed that a single administration of reovirus significantly reduced tumor size (Mann-Whitney test, \( P = 0.0159 \); data not shown) and also prolonged the survival of virus. In experiments comparing survival, all (9 of 9) live virus-treated animals were dead or had to be sacrificed by day 32, whereas 7 of 10 (70%) live virus-treated animals died of tumor growth by day 41. Three of 10 (30%) live virus-treated animals were long-term survivors that lived up to 169 days when the experiment was arbitrarily terminated. Histologic analysis showed all (9 of 9 = 100%) dead virus-treated animals were dead or had to be sacrificed by day 32, whereas 7 of 10 (70%) live virus-treated animals died of tumor growth by day 41. Three of 10 (30%) live virus-treated animals were long-term survivors that lived up to 169 days when the experiment was arbitrarily terminated. Histologic analysis showed all (9 of 9) dead virus animals and 70% (7 of 10) of live virus-treated animals had huge tumors; no tumor or microscopic tumor was found in the brain of the three long-term survivors.

**Presence of Circulating Reovirus Antibody Did Not Significantly Affect Survival.** Because the majority of the normal population is believed to have reovirus antibodies (27) and reovirus-neutralizing antibodies may limit reovirus’ therapeautic effect (7, 28), we tested the antitumor activity of reovirus against gliomas in immunocompetent animals that had circulating neutralizing reovirus antibody. We tested this in both s.c. and intracranial glioma animal models. We raised and confirmed the presence of neutralizing reovirus antibody for the following experiments. Reovirus-neutralizing antibodies were detectable 2 weeks after i.p. inoculation of reovirus in all animals (data not shown). In the s.c. 9L tumor model, reovirus was still effective in inhibiting tumor growth in animals previously exposed to reovirus (two-way ANOVA, \( P = 0.0011 \)). Compared with rats that received a single viral administration, animals that received multiple administrations of reovirus survived significantly longer (log-rank test, \( P < 0.0007 \); Fig. 3A). We therefore used multiple intracranial administrations (i.e., three injections) of reovirus in the subsequent therapeutic studies (we did not use more than three administrations because it seemed impractical from a clinical perspective).

We found similar results in the intracranial 9L model where tumor growth was significantly suppressed (Mann-Whitney test, \( P = 0.0022 \); Fig. 3B) and survival prolonged (log-rank test, \( P < 0.0016 \); Fig. 3C) with live virus intratumoral treatment. In experiments comparing tumor size, the tumors occupied 28.8% of the brain in coronal sections in the dead virus group compared with 3.8% in live virus group. In experiments comparing survival, all (9 of 9 = 100%) dead virus-treated animals were dead or had to be sacrificed by day 32, whereas 7 of 10 (70%) live virus-treated animals died of tumor growth by day 41. Three of 10 (30%) live virus-treated animals were long-term survivors that lived up to 169 days when the experiment was arbitrarily terminated. Histologic analysis showed all (9 of 9) dead virus animals and 70% (7 of 10) of live virus-treated animals had huge tumors; no tumor or microscopic tumor was found in the brain of the three long-term survivors.

**Fig. 3** Intratumoral administration of reovirus significantly inhibited tumor size and prolonged survival of rats with intracerebral raceine gliomas. A. Survival of animals bearing RG2 tumor in their brains was significantly prolonged by reovirus (log-rank test, \( P < 0.0011 \)). Compared with rats that received a single viral administration, animals that received multiple administrations of reovirus survived significantly longer (log-rank test, \( P < 0.0007 \); DV1 versus DV3, \( P = 0.235 \); DV3 versus LV3, \( P = 0.0007 \)); DV1 = single treatment with DV; LV3 = three administrations of LV. B. Reovirus significantly reduced tumor size of animals bearing 9L tumors (Mann-Whitney test, \( P = 0.0022 \)). C. Significantly prolonged survival (log-rank test, \( P < 0.0016 \)) was also found in animals with intracranial 9L glioma tumors. The median survival of the LV-treated animals (40 days) was significantly longer than DV animals (27 days).
animals with either 9L or RG2 tumors implanted intracranially (Fig. 4, A and B). No significant difference was found between survival of live virus-treated immunized mice and that of naïve (antibody negative) animals (log-rank test, P = 0.5586; Fig. 4A). Similar results were found in animals bearing intracranial RG2 tumors: reovirus was found to prolong survival of both naïve (log-rank test, P = 0.0011) and immunized animals (log-rank test, P = 0.0062), but no statistically significant difference was found between survival of live virus-treated immunized and naïve animals (median survival of live virus-treated immunized or naïve animals was 23 and 25 days; log-rank test, P = 0.2999; Fig. 4B). There are trends for naïve rats to have longer survival compared with the immunized counterparts in experiments with both models. On the basis of these trends, we next examined whether suppression of circulating antibodies might enhance reovirus efficacy.

Immunosuppression with Cyclosporine Did Not Improve Survival of Rats with Racine Intracranial Gliomas after Reovirus Administration. On the basis of the above observations that there was a trend toward longer survival in antibody-negative animals and our previous findings that reovirus’ antitumor activity can be enhanced by immune suppression in immunocompetent animals (7), we proposed that the efficacy of reovirus in brain tumors might be improved by immunosuppression with cyclosporine. To test this theory, we raised circulating neutralizing antibodies by i.p. administration of reovirus, followed by implantation of racine gliomas intracranially, then treated them with either dead virus or live virus alone or in combination with the immunosuppressant cyclosporine. The levels of circulating reovirus antibodies were determined again 16 days after reovirus treatment. Serial administration of cyclosporine successfully suppressed reovirus antibody levels in rat serum (Fig. 5A) and also decreased CD8 positive T-cell infiltration in the tumor (data not shown). However, combination of cyclosporine and reovirus did not improve survival of animals bearing 9L tumors (log-rank test, P = 0.6867; Fig. 5B). No statistically significance was found between immunized group and nonimmunized group treated with reovirus and cyclosporine (log-rank test, P = 0.7649). Similar results were found when we used animals implanted with RG2 allografts (Fig. 5, C and D). Neutralizing reovirus antibody levels were significantly decreased in immunized animals by cyclosporine (Fig. 5C), whereas the survival of these animals was not significantly improved (log-rank test, P = 0.2609; Fig. 5D).

Reovirus Inhibited Tumors in the Ipsilateral Hemisphere but not Remote Tumors in the Contralateral (Noninoculated Side) in Immunocompetent Rats; Reduced Distribution of Reovirus May Account for Its Inability. Because the major barrier to effective glioma therapy is the ability to target remote, invasive glioma cells, we assessed the ability of reovirus to infect remote intracranial tumors with only one (ipsilateral) inoculation. We implanted both hemispheres of the brain with RG2-GFP cells and intratumorally injected only the ipsilateral tumor with reovirus after the allografts were established. Tumor size was measured 12 days after viral infection. Significant tumor suppression was found only in the live virus-treated ipsilateral tumor (Mann-Whitney test, P = 0.0013) but not in the contralateral (Mann-Whitney test, P = 0.7546) tumor (Fig. 6A). In a similar but separate experiment, animals were sacrificed at different time points after reovirus administration, and GFP-labeled tumors were dissected under fluorescent imaging and tested for reovirus infection by viral culture, immunohistochemistry, and in situ hybridization. Reovirus was recovered from homogenates of live virus-treated tumors, including both the ipsilateral and contralateral sides, with the ipsilateral tumors having significantly higher (15- to 330-fold) titers than the contralateral tumors over the entire period of observation (Fig. 6B). Reovirus was not found in dead virus-treated tumors (data not shown). Results of immunohistochemistry and in situ hybridization reflected titers of reovirus within tumors shown in Fig. 6B. Reovirus mRNA and protein were present in ipsilateral tumors up to 48 hours after viral inoculation but were barely detectable in contralateral tumors or ipsilateral tumors 96 hours after infection (representative pictures are shown; Fig. 6C). The data suggest that dramatically smaller
Neutralizing reovirus antibody levels were significantly decreased (intracranial titer were 1/15 to 1/330 less than those in the ipsilateral tumors) amounts of virus reaching the contralateral tumors may account for its inability to kill these remote tumors. We also evaluated the spread of reovirus to the serum and found reovirus was present (~1.8 × 10^7 PFU/mL; via viral culture) in serum of live virus animals within 2 hours of virus inoculation; no virus was detected in the serum of live virus-treated rats at later time points or dead virus-treated animals (data not shown).

Mild Histologic Abnormalities and No Behavioral Changes Were Found in Fischer 344 Rats Intracerebrally Given Therapeutically Effective Doses of Reovirus (i.e., 10^9 PFU, Given Three Times). Because previous reports found reovirus was safe when given intracranially to adult immunocompetent animals (26), we were surprised that very high doses (4 × 10^9 PFU, five times) were lethal. We therefore evaluated the safety of the dose we used therapeutically in detail. We used both a behavioral test and histopathological analysis. Morris water maze testing showed that all animals (i.e., nonoperated controls, dead virus- and live virus-treated animals) had normal performances and went through a regular learning curve. Almost all of the rats failed to locate the platform (latency > 90 seconds) in the first trial but learned at a normal rate thereafter, and eventually, >90% of them successfully located the platform within ~20 seconds by the twentieth trial (Fig. 7A, representative swimming paths, top two rows). Starting from trial 21, the platform was placed in the diagonally opposite quadrant of the pool (as a control) and another four trials were done. Rats of all groups searched intensively for the platform in the previously correct quadrant (Fig. 7A, bottom row), suggesting that learning occurred by using surrounding environmental cues. The latency decreased for all groups over five trial blocks (1 to 20th trials) and appropriately increased during the 6th block (i.e., 21 to 24th trials) when the platform was moved. No difference was found in average latency between normal (i.e., nonoperated controls), dead virus- and live virus-treated groups (two-way ANOVA, P > 0.976; Fig. 7B). The experiment was repeated, and similar results were obtained.

Histologic analysis showed rats given live virus had communicating hydrocephalus, subependymal inflammation, and signs of mild local inflammation at the site of viral inoculation (Fig. 7C). These changes were not seen in the dead virus-treated or control (i.e., nonoperated) animals (data not shown). Localized calcification and activated inflammatory responses (microglia, macrophages, lymphocytes, and so on) were evident around the injection site. Slightly dilated ventricles were found in most live virus-treated brains. No aqueductal obstruction or obstruction elsewhere in the cerebrospinal fluid pathways was found in animals with hydrocephalus. The brains of dead virus-treated or control animals were normal. To additionally characterize any diffuse inflammatory response (not evident in H&E staining) and gliosis, we examined the brains after staining with glial fibrillary acidic protein, and no difference was found (log-rank test, P = 0.0154, P = 0.0091). Again, CyA did not prolong survival (log-rank test, P = 0.7801), and naïve animals treated with CyA plus LV had a slightly longer survival compared with reovirus-immunized animals in the LV + CyA group (LV + CyA versus LV, P = 0.2609).
between dead virus- and live virus-treated brains. For example, normal hippocampal structures and astrocyte morphology were found in all animals (data not shown). No reactive astrocytes or changes in neuronal density or morphology were observed in any region of the brains of live virus-treated animals. Similarly, there was no evidence of diffuse encephalitis. In addition, no abnormality was found in major organs (i.e., liver, kidneys, heart, lungs, and spleen) other than the brain. Reovirus antibodies, but not reovirus mRNA or protein, were detectable in the serum and brain of the live virus-treated animals 120 days after viral infection (data not shown).

**Intracerebral Inoculation of Reovirus in Cynomolgus Monkeys Was Well Tolerated.** General information and laboratory examination results of the primate studies are summarized in Table 1. There were no unscheduled deaths during the study. Transient clinical signs, including convulsions followed by lying on cage floor and increased respiration, were observed once on day 6 in the male monkey received the highest dose of reovirus (i.e., $1.51 \times 10^9$ PFU). Potential treatment-related slight increases in rectal temperature (ranging from 39.6°C to 40.3°C) were observed in the highest dose group during the first week of treatment/observation period (rectal temperatures ranging from 37.5°C to 39.5°C were considered as normal). Over the course of the study, there was no treatment-related effect on body weight or food intake.

The hematology and cell morphology analysis of blood samples from all animals revealed no effect of treatment with reovirus. Measurements of activated partial thromboplastin time and of prothrombin time revealed no evidence that the administration of reovirus had any effect on blood clotting. There were no changes in clinical chemistry data that could be attributed to treatment with reovirus. The urinalysis and the measurement of urinary volume and specific gravity revealed no evidence that suggested an effect of reovirus. Organ weight data obtained from the termination revealed no evidence of an effect of reovirus.

Samples were examined for viable virus by viral culture and presence of reovirus RNA by RT-PCR analyses for the S3 gene. Viral shedding data are summarized in the Table 2. Evidence of viral shedding (positive in both viral culture and RT-PCR) was found in urine of two (2 of 8 = 25%) monkeys (one female in the low dose group and one male in the high dose group). Reovirus s3 RNA was detected occasionally in excreta (i.e., serum, cerebrospinal fluid, feces, and nasal) of six other animals, including two monkeys from the control group. Reovirus antibodies were present in serum or cerebrospinal fluid of monkeys who received reovirus but not in control animals tested.

The microscopic findings were limited to the injection site (brain) and all other findings were considered incidental. The findings of the brain after 8 weeks are consistent with a previous area of necrosis undergoing active healing to form an astrocytic scar. The sizes of the lesions increased with dose and exceed the trauma expected from the experimental procedure. The changes seen in most animals after 6 months were consistent with healing of a localized injury to the cortex and appear to be...
The microscopic changes were dose-related and exceed the trauma expected from the dose administration procedure. Animals treated with saline had only local changes attributable to the trauma of the stereotaxy [i.e., focal, superficial astrogliosis with minimal chronic inflammation (Fig. 8A) and hemosiderin deposition]. At the lowest dose of reovirus (1.51 \times 10^6 PFU), mild reactive changes in the region of viral administration were seen (Fig. 8B). These included mild chronic inflammation both in the brain parenchyma and the contiguous subarachnoid space, focal subarachnoid hemorrhage, and chronic cicatrical lesions with mild astrogliosis, scattered foamy macrophages, and hemosiderin deposition. Monkeys treated with the intermediate doses (1.51 \times 10^7 to 1.51 \times 10^8 PFU) of reovirus had moderate reactive changes. These changes included a diffuse chronic meninitis, deposition of hemosiderin, moderate lymphocytic perivascular space cuffing with spillover into the white matter substance, edema, and localized subacute infarctions with foamy macrophage deposition and moderate astrogliosis around the needle tracks (Fig. 8, C and D). At the highest dose of reovirus (1.51 \times 10^9 PFU), lesions were found in only one of the two animals treated with this dose (despite multiple samplings of brain tissue). In this animal, severe acute and chronic reactive changes were present at the injection site consisting of diffuse surface injury with subpial inflammation, neovascularization, astrogliosis, subacute infarction with abundant foamy macrophages, and collections of axonal spheroids, connective tissue desmoplasia, and marked chronic inflammation in the brain parenchyma and perivascular spaces (Fig. 8, E and F). The brain of the other animal treated with the highest dose of reovirus was normal. In terms of brain regions remote from the site of viral administration, all animals (i.e., both saline and reovirus treated) had mild-to-moderate acute and chronic Purkinje cell hypoxic damage and resultant Bergmann gliosis in the cerebella (data not shown). Additionally, most animals also had focal acute petechial hemorrhages in the gray matter of the spinal cords of unclear etiology.

**DISCUSSION**

This study illustrates the potential usefulness and minimal toxicity of a naturally occurring replication competent virus that alternative strategies by the rats, the platform was moved to the diagonally opposite quadrant (i.e., 21st to 24th trials or 6th trial block). As expected, all animals searched intensively for the now misplaced platform. Rats of the control, DV and LV groups had similar patterns of searching tracks. B. Average latency to finding the platform decreased normally and was similar in the three groups (nonoperated, n = 7; DV treated, n = 8; LV treated, n = 8). Each trial block represents the average of four individual trials. There was no difference in the performance between groups (two-way ANOVA test, P > 0.976). As a control, after the fifth trial block, the platform was placed in the diagonally opposite quadrant, and as expected, an increase in latency occurred in all three groups. C. Histologic changes in the brain of LV-treated animals show mild communicating hydrocephalus. There were infiltrating lymphocytes along the ependymal and subependymal layer of the ventricles (top left). Calcification (arrowheads), activated microglia, and lymphocytes were present locally in the area of viral inoculation [top right; original magnifications, \( \times 40 \) (top) and \( \times 200 \) (bottom)]. Slightly dilated ventricles were found in most LV-treated animals but not in nonoperated or DV-treated animals (bottom row).
kills glioma cells and prolongs survival in immunocompetent racine models of malignant glioma. We found only localized toxicities when reovirus was introduced into the brains of Cynomolgus monkeys. Although our previous work has shown the mechanism whereby reovirus selectively infects and kills tumor cells (16, 4) and that reovirus is effective in experimental models of brain tumors (5, 12), these experiments were all done in immunocompromised hosts (e.g., SCID and nude mice).

Because the host immune response is probably critical in terms of the efficacy and toxicities of oncolytic viruses, we considered these experiments in rats and monkeys important steps in the evaluation of reovirus as an experimental therapeutic agent in glioma patients.

It is important to evaluate any potential oncolytic virus in immunocompetent hosts in which innate and acquired immune responses to the virus more closely mimic those encountered in the human body. This can be achieved by using immunocompetent animal models such as Cynomolgus monkeys, which more closely resemble humans in terms of immune function compared to immunocompromised models. The use of immunocompetent models allows for a better understanding of the virus’s efficacy and potential toxicities in a more relevant setting.

### Table 1: Summary of Cynomolgus monkeys inoculated with reovirus intracranially

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Gender (M/F)</th>
<th>Virus dose (PFU)</th>
<th>Time until sacrifice (mo)</th>
<th>Fever</th>
<th>Seizure</th>
<th>Lab exam</th>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1501A</td>
<td>F</td>
<td>0</td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1001B</td>
<td>M</td>
<td>0</td>
<td>6</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-dose group</td>
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</tr>
<tr>
<td>2501A</td>
<td>F</td>
<td>$1.51 \times 10^6$</td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001B</td>
<td>M</td>
<td>$1.51 \times 10^6$</td>
<td>6</td>
<td>+</td>
<td>Normal</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3501A</td>
<td>F</td>
<td>$1.51 \times 10^7$</td>
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<td>Normal</td>
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<tr>
<td>3001B</td>
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<td>$1.51 \times 10^7$</td>
<td>6</td>
<td>Normal</td>
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<td></td>
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<tr>
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<tr>
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<td>F</td>
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<td>Normal</td>
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<td></td>
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<tr>
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<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-dose group</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5501A</td>
<td>F</td>
<td>$1.51 \times 10^9$</td>
<td>3</td>
<td>+</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>5001B</td>
<td>M</td>
<td>$1.51 \times 10^9$</td>
<td>6</td>
<td>+</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; time until sacrifice, time from viral inoculation until sacrifice; lab exam, clinical laboratory examination, including hematology, coagulation profile, chemistry, and urinalysis; fever and seizure: only listed if present (+).

### Table 2: Viral shedding of Cynomolgus monkeys inoculated with reovirus intracranially

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Serum PCR</th>
<th>Cerebrospinal fluid PCR</th>
<th>Feces PCR</th>
<th>Urine PCR</th>
<th>Nasal PCR</th>
<th>reo antibodies (sac.)</th>
</tr>
</thead>
<tbody>
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<td>Control group</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1501A</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- w - - nd</td>
<td>(d15)</td>
</tr>
<tr>
<td>1001B</td>
<td>- - w (d20)</td>
<td>- - nd</td>
<td>- - nd</td>
<td>- - nd</td>
<td>- - nd</td>
<td>-</td>
</tr>
<tr>
<td>Low-dose group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2501A</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- w w (d15)</td>
<td>- - - nd</td>
<td>nd</td>
</tr>
<tr>
<td>2001B</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>nd - nd</td>
<td>- - nd</td>
<td>nd</td>
<td>256</td>
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<tr>
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<td>- - - nd</td>
<td>- - - nd</td>
<td>nd - w</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>nd</td>
</tr>
<tr>
<td>3001B</td>
<td>- - - nd</td>
<td>w - - (d10)</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>nd</td>
</tr>
<tr>
<td>Moderate high-dose group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4501A</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>nd</td>
</tr>
<tr>
<td>4001B</td>
<td>- - - nd</td>
<td>w - - (d180)</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>128</td>
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<td></td>
</tr>
<tr>
<td>5501A</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>w - - nd</td>
<td>(d2)</td>
</tr>
<tr>
<td>5001B</td>
<td>- - - nd</td>
<td>- - - nd</td>
<td>nd</td>
<td>w - - nd</td>
<td>+ w - - nd</td>
<td>(d2)</td>
</tr>
</tbody>
</table>

NOTE. Numbers in antibody column are reovirus antibody titers.

Abbreviations: cult, viral culture; sac, at the time of sacrifice; d2, d15, d20 = days 2, 15, and 20 after viral inoculation, respectively; -, negative; nd, not done; w, weakly positive; +, positive.
clinically. This is particularly true because the immune response could have a variety of effects that could be theoretically either beneficial or harmful. An example of a beneficial effect would be enhancing the oncolytic effect by an immune-related inflammatory response within the tumor. This has led to a strategy to introduce cytokines into some oncolytic viruses (29–33) to improve tumor cell killing. On the other hand, immune responses may reduce an oncolytic virus' therapeutic efficacy by reducing the amount of virus that reaches the tumor or by causing serious side effects such as chronic brain inflammation (34). There have been a number of studies evaluating the efficacy of several oncolytic viruses in immunocompetent hosts (7, 28–30, 34–39) but few have been in gliomas (28, 29, 34, 38, 39). Here, we show that the intracerebral administration of reovirus significantly prolongs survival in an immunocompetent model of malignant gliomas. We are aware that part of the therapeutic response seen in one model (i.e., 9L) may be due to its immunogenicity (40). However, the prolonged survival achieved with RG-2 [which is syngenic and lacks any immunogenic effects (40)] supports a direct oncolytic effect. Despite several publications (28, 39, 41–46) that use 9L as a tumor model to test a variety of therapeutics, we have now abandoned its use for our own future therapeutic studies.

Because we propose to use this live naturally occurring virus and administer it directly into the brains of patients with malignant gliomas, an evaluation of its toxicity is critical. We were surprised by toxicities in racine models but reassured by a lack of significant toxicities in the nonhuman primates. High enough doses of reovirus administered into racine brains were lethal. Of course reovirus is well known to be lethal in newborn (47) or severely immunocompromised animals [e.g., SCID mice (4)] but has previously been reported as safe in immunocompetent adult animals, even when administered intracerebrally [up to $5 \times 10^9$ PFU given once intracranially to rats (26); up to $5 \times 10^8$ PFU given once intracranially to mice (47)]. In contrast, we found that very high doses of virus given frequently (e.g., $4 \times 10^9$ PFU given five times) produced diffuse encephalitis, whereas the therapeutically effective dose we used of $1 \times 10^9$
PFU produced only a mild communicating hydrocephalus. Importantly, behavioral tests showed that therapeutically effective doses of reovirus do not cause impairment in animals.

The lack of diffuse abnormalities in the brains of Cynomolgus monkeys after high doses of reovirus was reassuring. In the primate brain the changes were confined to the region of viral inoculation and were dose related. At the highest dose (1.51 × 10^7 PFU), there was a microscopic area of subacute infarction at the site of viral inoculation. The focal changes we found were apparently more marked than those reported when the attenuated herpes simplex virus G207 was inoculated into the brains of nonhuman primates (24, 25). Whether small areas of focal infarction will be clinically significant in patients with large regions of necrotic tumor is unknown, and a definite answer awaits the completion of the clinical trial in patients with recurrent malignant gliomas. We are not sure what accounts for the difference in toxicities we found in rats as opposed to primates. It could be due to a species difference in susceptibility to reovirus infection or to a contaminant in our laboratory stocks of reovirus we used in rats. The reovirus used in the primate study was clinical grade and prepared to Good Manufacturing Practice guidelines under contract at an independent laboratory and is free of contaminants. In contrast, the reovirus used in the racine studies was prepared in our laboratories under nonsterile conditions. Accordingly, it could contain contaminants that might produce the toxicities we found in rats. Finally, from a practical perspective, because one of the monkeys had a generalized seizure after intracerebral inoculation, patients with recurrent malignant gliomas being treated with reovirus (phase I/II trial is in progress; P. Forsyth is principal investigator) are treated with prophylactic anticonvulsants to prevent seizures from occurring.

Another important feature of an oncolytic virus’ safety is shedding into body secretions because this could impact the health of people in contact with the patient such as health careworkers, family members, or the public at large. We did find viral shedding in the urine of two monkeys but do not consider this clinically important because the virus is benign, not known to cause disease, and most people have been infected previously. On the other hand, shedding with a highly virulent or genetically unstable virus would be a potentially serious public health issue. Because the virus is benign, we only require patients treated with reovirus to wear a mask and avoid contact with infants and severely immunocompromised individuals for 2 weeks after viral inoculation.

We have not definitely determined whether manipulation of the immune system can be used to alter the efficacy of this approach in the intracranial administration of reovirus. We were limited by the unavailability of Good Manufacture Practice prepared because this has been earmarked for clinical trials. Without a Good Manufacture Practice virus, our experiments could be confounded by the potential contaminants in the laboratory stock of virus. Nevertheless, we make several conclusions. (a) The presence of circulating neutralizing reovirus antibodies, which we will anticipate would be present in the majority of patients, does not seem to affect the response to this treatment in terms of efficacy or toxicity. (b) Immunosuppression with cyclosporin [which we show reduces CD8 T-cell infiltration into the tumor (data not shown), as well as levels of antibodies] did not enhance survival after reovirus treatment. Cyclosporin A does penetrate the blood brain barrier whether or not a lesion is present (48–50). Cotreatment with immunosuppression is effective in systemic delivery and enhances the therapeutic effect of oncolytic viruses (28, 51), including reovirus (7). We were surprised that we did not find evidence of viral replication in these immunocompetent models of brain tumors in which reovirus was directly inoculated (i.e., Figs. 2C and 6B) but did find some viral replication in the contralateral, noninoculated tumor when grown in the brain. However, viral infection with release of viral progeny and iterative infection of adjacent tumor cells occurs in immunocompromised models of tumors outside of the brain (10). We speculate that reduced viral replication in this setting is because of an immune response to the virus. In future experiments, when Good Manufacture Practice-grade reovirus is available, we will first characterize the immune response to the intracerebral administration of reovirus and then manipulate components of the immune system (e.g., by selective T-cell depletion, macrophage/microglia suppression, complement depletion, and so on) to determine whether we can improve viral replication and the efficacy of this approach in immunocompetent hosts.

The ability to infect and kill remote (invasive or metastatic) tumor cells is critically important in the treatment of cancer (52, 53) and in gliomas in particular. Gliomas are highly invasive tumors that extend infiltrative tendrils of tumor into the surrounding normal brain and render them surgically incurable. This has led to efforts to target the invasive tumor cells in addition to the main tumor mass. We found in an immunocompromised s.c. model of malignant gliomas that reovirus would infect and kill remote contralateral, noninoculated tumors (4) and therefore tested if this was possible in immunocompetent models. Reovirus did not cause regression of gliomas in the contralateral hemisphere in the brain. Although on the one hand this may seem discouraging, it is more remarkable that virus is present in contralateral brain tumors at such high levels! On average, there was only a 1.9-log difference (i.e., 84-fold difference) in the viral titers compared with the ipsilateral tumors, which were directly inoculated with reovirus (e.g., ~7.8 × 10^3 versus 1.6 × 10^2 PFU at 96 hours after infection). Although (somewhat surprisingly) we do not know if there is a dose-response relationship with reovirus (or with other oncolytic viruses), we suspect that there will be one in immunocompetent hosts in which the immune system may limit the spread/distribution of the virus within the tumor or to remote sites. Therefore, reovirus can infect remote glioma tumors at very high viral titers. Given the relatively high levels of virus present in the contralateral tumor, it suggests the possibility that manipulation of the immune system or alternative models of drug delivery (i.e., manipulation of the blood brain barrier) may enhance delivery to remote tumors. These experiments are under way in our laboratory.

When combined with our previous studies (4, 5, 12, 13) we used these data regarding efficacy in gliomas and safety in primates to construct a phase I/II study of reovirus in patients with recurrent malignant gliomas; this trial is still in progress. We have already completed a phase I study of reovirus in patients with cutaneous metastases from systemic cancer. We found it safe and several responses were seen (54). As such, we
awaits the results of our clinical trial in patients with recurrent malignant gliomas to definitively determine how safe reovirus is to administer in the brain of patients and to obtain preliminary indications of its efficacy.

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Efficacy and Safety Evaluation of Human Reovirus Type 3 in Immunocompetent Animals: Racine and Nonhuman Primates

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