

Attenuated, Replication-Competent Herpes Simplex Virus Type 1 Mutant G207: Safety Evaluation in Mice

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Herpes simplex virus type 1 (HSV-1) mutants that are attenuated for neurovirulence are being used for the treatment of cancer. We have examined the safety of G207, a multimutated replication-competent HSV-1 vector, in mice. BALB/c mice inoculated intracerebrally or intracerebroventricularly with 10⁷ PFU of G207 survived for over 20 weeks with no apparent symptoms of disease. In contrast, over 80% of animals inoculated intracerebrally with 1.5 × 10³ PFU of HSV-1 wild-type strain KOS and 50% of animals inoculated intracerebroventricularly with 10⁴ PFU of wild-type strain F died within 10 days. Similarly, after intrahepatic inoculation of G207 (3 × 10⁷ PFU) all animals survived for over 10 weeks, whereas no animals survived for even 1 week after inoculation with 10⁶ PFU of KOS. After intracerebroventricular inoculation, LacZ expression was initially observed in the cells lining the ventricles and subarachnoid space; expression decreased until almost absent within 5 days postinfection, with no apparent loss of ependymal cells. G207 DNA could be detected by PCR in the brains of mice 8 weeks after intracerebral inoculation; however, no infectious virus could be detected after 2 days. As a model for latent HSV in the brain, we used survivors of an intracerebral inoculation of HSV-1 KOS at the 50% lethal dose. Inoculation of a high dose of G207 at the same stereotactic coordinates did not result in reactivation of detectable infectious virus or symptoms of disease. We conclude that G207 is safe at or above doses that were efficacious in mouse tumor studies.

Herpes simplex virus type 1 (HSV-1) is a neurotropic DNA virus which infects a wide range of cell types in different animals. Natural infections either follow a lytic, replicative cycle or establish latency. Latency is characterized by the long-term persistence of viral DNA in latently infected neurons, the lack of infectious or replicating virus, the lack of viral gene expression except for the latency-associated transcripts (LATs), and in some situations episodic reactivation of infectious virus (74). In humans, the natural host, HSV-1 causes a number of diseases, including gingivostomatitis and pharyngitis after primary oral-facial infection, recurrent herpes labialis, genital herpes, keratitis after eye infection, disseminated visceral infections in immunocompromised patients, hepatitis, and encephalitis after spread to the central nervous system (CNS) (13).

HSV encephalitis is the most common CNS viral infection, occurring in two to three persons per million (13). Brain pathology is usually localized to the temporal lobe and limbic system, with asymmetric necrotizing encephalitis, inflammation, and hemorrhage (19, 33). The majority of cases of encephalitis occur in patients with recurrent HSV who are seropositive at onset of disease. Even with acyclovir therapy, mortality is about 20%, and about 20% of survivors have long-term morbidity, including cognitive abnormalities (23, 51).

A number of animal models of HSV encephalitis involving spread from peripheral infection (3, 80) or direct inoculation in the CNS (11, 39, 50) have been developed. Direct inoculation

into the olfactory bulb in rabbits and rats leads to encephalitis, with virus isolatable from the temporal cortex (69) and surviving animals exhibiting learning deficits (3, 53). In addition, encephalitis has been induced in rabbits by reactivation of latent HSV established by intranasal inoculation of a neurovirulent strain (75). About a quarter of the reactivated rabbits developed behavioral changes (lethargy and unresponsiveness), and a similar proportion had gross necrotic lesions in the cortex within 1 to 2 weeks postreactivation (75).

Considerable progress has been made in understanding latency in the sensory ganglia of the peripheral nervous system (PNS). Unfortunately, latency in the CNS is much less characterized. The role of CNS latency in pathogenesis has become an important issue with the demonstration of HSV DNA in human brain tissue from patients without encephalitis. HSV DNA was detected in brains from 14 of 40 HSV-seropositive patients, mostly in the olfactory bulb, pons, and medulla, (2), and from 6 of 22 patients with nonneurologic disease and 5 of 22 patients with Alzheimer's disease (68). PCR primers in these studies could not differentiate HSV-1 from HSV-2. In a study of 109 human corpses at forensic postmortem, 16% were positive for HSV-1 DNA in the olfactory bulbs, whereas 72% of the trigeminal ganglia were positive (43). In animal models, HSV DNA can often be detected in the CNS of animals surviving HSV-induced encephalitis (17, 67).

Attenuated replication-competent mutants of HSV-1 have been shown to be efficacious in the treatment of a variety of experimental tumor models (1, 5, 6, 30, 49, 56, 63, 65, 88). We have created a second-generation, multimutated HSV-1, G207, that is currently in clinical trial for the treatment of recurrent gliomas. G207 has deletions of both copies of the γ 34.5 gene (RL1) and an *Escherichia coli lacZ* insertion that inactivates the ICP6 gene (UL39) (57). Mutations in γ 34.5 causes a large decrease in neurovirulence, so that after intracerebral (i.c.) inoculation in mice, the 50% lethal dose (LD₅₀) is >10⁷ PFU

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TABLE 1. Primer pairs used in this study

Name ^a	Gene	Primer sequence ^b	PCR product size (bp)	Reference
RR (ICP6)	UL39	5' GACAG CCATA TCCTG AGC 3' 3' GCCAG CAGTT GCTAG AACT CA 5'	221	15
TK	UL23	5' CTGCA GATAC CGCTC CGTAT T 3' 3' TACCC TACCG CCAGC TTCTA C 5'	273	46
gE	US8	5' GAGATGCGAATATACGAAT 3' 3' TAAAC CGGCT CGGGT GGGTG 5'	320	18
LacZ		5' TTGCT GATTC GAGGG GTTAA CCGTC ACGAG 3' 3' TAGCA TTAGT GCGGT CACAC TAGTAG ACCA 5'	300	64
Fx		5' AGACT CTTAC CTTTG GGGAT A 3' 3' TCGAT TACTT CGTGA CCAGT G 5'	127	40

^a Fx is a single-copy mouse gene, RR, TK, and gE amplify HSV-1 sequences, and LacZ is unique to G207.

^b Sequences listed 5' to 3' are upstream primers; 3'-to-5' sequences are downstream primers.

within the standard 3-week observation period (10, 47). In prior studies, we were unable to detect an LD₅₀ for G207 after i.c. inoculation in mice or *Aotus nancymae* primates (25, 57). However, as part of the preclinical evaluation of these vectors for use in tumor therapy, we expanded on these studies and examined other potential pathological consequences of G207 inoculation. Other safety concerns include inoculation into the ventricles of the brain, leakage of virus into the bloodstream, or infection of susceptible peripheral organs. Another issue to consider is whether i.c. inoculation of G207 could lead to reactivation of, or complementation or recombination with, a patient's latent virus to cause disease. It has been shown in mice that mixed infections with two nonlethal nonneuroinvasive HSV-1 strains can result in a lethal infection and spread to the brain (29). To address this, we used a mouse model involving i.c. inoculation of wild-type HSV-1 strain KOS and subsequent challenge of the survivors with a high dose of G207 at the same i.c. location. The present studies found that G207 caused no detectable disease in mice at titers many logs above the lethal dose of the parental virus, HSV-1 strain F.

MATERIALS AND METHODS

Viruses. HSV-1 G207 (57), parental wild-type strain F (obtained from B. Roizman, University of Chicago, Chicago, Ill.), wild-type strain 17syn⁺ (obtained from J. Subak-Sharpe, Institute of Virology, Glasgow, United Kingdom), and wild-type strain KOS1.1 (obtained from D. Knipe, Harvard Medical School, Boston, Mass.) were propagated on mycoplasma-free Vero (African green monkey kidney) cells (American Type Culture Collection). Virus stocks were prepared by infecting subconfluent monolayers of Vero cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% heat-inactivated fetal calf serum (IFCS) (HyClone, Logan, Utah) at a multiplicity of infection of 0.01. Infected cells were incubated at 34 or 37°C and harvested when total cytopathic effect was observed. After a freeze-thaw/sonication regimen, cell debris was removed by low-speed centrifugation (2,000 × g at 4°C for 10 min) and virus (F and G207) was concentrated by high-speed centrifugation (45,000 × g at 4°C for 60 min). The viral pellet was resuspended in virus buffer (VB; 150 mM NaCl, 20 mM Tris [pH 7.5]) and titered by plaque assay on Vero cells. Viral stocks were stored at -80°C and thawed rapidly for use. Mock extracts were prepared identically, except that VB was used in place of virus during the infection step.

Intracerebral inoculation of HSV-1. Three-week-old BALB/c female mice, obtained from the National Cancer Institute (Frederick, Md.), were anesthetized with a 250-μl intraperitoneal (i.p.) injection of sodium pentobarbital solution (84% bacteriostatic saline, 10% sodium pentobarbital [50 mg/ml; Abbott Laboratories, Chicago, Ill.], 6% ethyl alcohol) and placed in a KOPF stereotaxic frame. A small burr hole was made 1 mm rostral of the bregma and 2 mm right of midline, and a beveled 29-gauge Hamilton needle was used to inoculate 2 to 5 μl of virus at a depth of 2 mm from the skull surface over a period of 10 min. After another 3 min, the needle was slowly withdrawn (26). Animals were monitored for 6 weeks to 1 year postinfection (p.i.). All procedures involving animals were approved by the Georgetown University Animal Care and Use Committee.

Three-week-old BALB/c female mice were inoculated i.c. with increasing amounts of KOS virus diluted in VB and monitored for 21 days. The PFU/LD₅₀ ratio was calculated (84). At 56 days p.i., a subset of surviving animals was

sacrificed and the presence of HSV DNA in the brain was determined by PCR. Sixty days after KOS inoculation, the surviving mice (5 × 10² to 20 × 10² PFU) were randomly divided into groups and inoculated i.c. at the same stereotaxic coordinates with 5 μl of G207 (10⁷ PFU) in VB or VB alone.

Intracerebroventricular (i.c.v.) inoculation of HSV-1. Ten- to twelve-week-old BALB/c female mice were anesthetized by i.p. injection of 250 μl of sodium pentobarbital solution and placed in a KOPF stereotaxic frame. A 2-mm burr hole was made 0.8 mm anterior and 1 mm off midline. A Hamilton microliter syringe containing G207 virus (10⁷ PFU) or mock extract in 10 μl was introduced to a 2-mm depth, and virus was injected slowly over 10 min. To confirm that an i.c.v. injection occurred, two mice in each group were injected with Evans blue (Sigma) dye (2% in saline) and sacrificed at the end of surgery.

For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) histochemistry, mice were anesthetized with 500 μl of sodium pentobarbital solution and perfused transcardially with cold 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, postfixed for 2 h in 4% paraformaldehyde, cut in half coronally, and stained with X-Gal substrate solution (56). Brain fragments were washed in PBS, cryoprotected overnight in 30% sucrose, sectioned on a cryostat at 10 or 40-μm thickness, mounted on gelatin-coated slides, and counterstained with carmalum or hematoxylin and eosin.

Intravenous (i.v.) inoculation of G207. Three-week-old BALB/c female mice were anaesthetized by i.p. injection of 250 μl of sodium pentobarbital solution. Virus (100 μl) was administered by percutaneous puncture of the lateral tail vein.

Intrahepatic (i.h.) inoculation of G207. Four- to five-week-old BALB/c mice were inoculated with 30 μl of virus or PBS into the right hepatic lobe as described elsewhere (58).

Titration of virus from the brain. Mice were sacrificed at different times following G207 challenge (1, 2, 4, 7, 14, and 30 days; two mice per time point) by lethal injection (500 μl of sodium pentobarbital solution). Brains were removed and quick-frozen. Brain homogenates were made in DMEM-10% FCS at a 10% wt/vol ratio, using a Thomas pestle tissue grinder (Thomas Scientific, Swedesboro, N.J.). After centrifugation at 700 × g at 4°C for 10 min, the supernatants were serially diluted in PBS-1% IFCS, and viral titer on Vero cells was determined.

PCR amplification. Brain tissue (~25 mg) was isolated from the right frontal cortex (inoculation site), and DNA was purified using a QIAamp tissue kit (QIAGEN, Chatsworth, Calif.) as described by the manufacturer. Amplification reactions were performed in 50-μl volumes containing DNA, 2.0 mM MgCl₂, 200 μM each deoxyribonucleoside triphosphate (Perkin-Elmer), 1 μM each primer (Gibco BRL, Gaithersburg, Md.), and 2.5 U of *Taq* polymerase (Perkin-Elmer) with PCR buffer II (Perkin-Elmer). Primer sequences and their expected product sizes are shown in Table 1. The conditions for PCR amplification were 94°C (denaturation) for 90 s, annealing at 55°C for 60 s, and extension at 72°C for 120 s (35 cycles) on a PTC-100 thermal controller (MJ Research Inc., Watertown, Mass.). Amplification products were separated by electrophoresis on a 2.5% NuSieve agarose (FMC) gel in Tris-acetate-EDTA (TAE) buffer and visualized by ethidium bromide fluorescence. To determine the sensitivity of the PCR assay, known amounts of virus was mixed with uninfected mouse brain, and DNA was purified and amplified as described above.

RESULTS

Safety of G207 in BALB/c mice. The safety of G207 inoculation in young BALB/c mice was determined after four different routes of inoculation: i.c., i.c.v., i.v., and i.h. The first three are likely sites of viral spread after inoculation of brain tumors in situ. Intracerebral inoculation of 10⁷ PFU of G207 or mock extract resulted in no symptomatic evidence of disease during the 1-year observation period (Table 2). In an earlier

TABLE 2. Mortality and morbidity of HSV in BALB/c mice after i.c., i.c.v., i.v., or i.h. injection^a

Virus	Dose (PFU)	Injection site	Survival (survived/injected)	Time to death (days p.i.)	Observation (wk)
VB		i.c.	12/12		8
Mock extract		i.c.	8/8		52
F ^b	1 × 10 ³	i.c.	4/8	3, 3, 5, 14	3
KOS	1.5 × 10 ³	i.c.	0/8	4, 4, 5, 5, 5, 5, 6, 7	
17syn ⁺	1 × 10 ²	i.c.	2/4	4, 5	3
G207	1 × 10 ⁷	i.c.	7/8	140 ^c	52
Mock		i.c.v.	8/8		28
F	1 × 10 ⁴	i.c.v.	4/8	7, 7, 7, 10	52
G207	1 × 10 ⁷	i.c.v.	10/11	168 ^d	28
VB		i.v.	4/4		52
G207	1 × 10 ⁷	i.v.	4/4		52
PBS ^e		i.h.	3/3		11
KOS ^e	1 × 10 ⁶	i.h.	0/4	2, 2, 6, 6	<1
G207	3 × 10 ⁷	i.h.	6/6		11

^a Animals were followed for the times indicated if no disease occurred. Where morbidity was observed, the animals were sacrificed or died at the days p.i. indicated (time to death).

^b Data from reference 57.

^c Unknown cause.

^d Sacrificed at 24 weeks due to neck abscess.

^e Data from reference 58.

set of studies from this laboratory (57), we found that 50% of mice died after an i.c. inoculation of 10³ PFU of strain F, similar to the reported LD₅₀ (10). The dose for i.c. injection of G207 was the maximum that could be administered in the small volumes required. No HSV-related mortality or morbidity was noted. Ten-week-old BALB/c mice were injected i.v. with G207 (10⁷ PFU) or mock extract and followed for 1 year with no evidence of disease (Table 2). HSV infection of the liver can cause a lethal necrotic hepatitis in both mice and humans (22, 32, 81). Direct injection of G207 into the liver at a very high dose (3 × 10⁷ PFU) caused no disease, whereas injection of 10⁶ PFU of KOS was uniformly lethal.

Intracerebroventricular injection of G207. Brain tumors are very often located near ventricles, and therefore it was important to determine the toxicity of G207 inoculation into the ventricles, where the virus could spread throughout the CNS. The accuracy of the i.c.v. injections was confirmed by injecting the first and last animal in a group with Evans blue dye, sacrificing the animal, and visualizing bilateral ventricular staining (see Fig. 2D). No symptoms of disease were noted in any of the animals inoculated with G207 (10⁷ PFU) or mock extract, whereas 50% of the strain F (10⁴ PFU)-inoculated animals died within 10 days (Table 2). The spread of G207 in the ventricular system was examined by sacrificing G207-injected mice on days 1, 3, and 5 p.i. and histochemically staining for LacZ expression (Fig. 1 and 2). In G207, the *lacZ* transgene is driven by the HSV-1 ICP6 promoter. The most intense X-Gal staining was seen 1 day p.i., in the bilateral ventricles and subarachnoid spaces (Fig. 1, d1; Fig. 2A). By day 3, the contralateral, left ventricle (Fig. 1, d3, left) is much less intensely stained, as are the subarachnoid spaces (Fig. 1, d3, right). There is almost no staining visible by day 5 (Fig. 1, d5; Fig. 2C) and no apparent loss of ependymal cells (Fig. 2F). Sectioning of these brains further demonstrated that LacZ expression was limited to cells in contact with cerebrospinal fluid (CSF) (Fig. 2). No X-Gal-stained cells of obvious neuronal morphology were detected. We have found that X-Gal staining is more sensitive than immunohistochemical staining for β-galactosidase (60).

Persistent HSV-1 KOS in the brain. Groups of eight 3-week-old female BALB/c mice were inoculated i.c. with doses of HSV-1 KOS ranging from 2.5 × 10² to 6 × 10³ PFU per mouse (Table 3). Most of the mice exhibited signs of CNS disease or encephalitis, such as ruffled fur, hunched posture, hind limb paralysis, and lethargy. Clinical symptoms in mice usually resolved within 48 h of onset, or death occurred. At the highest inoculation doses (3 × 10³ and 6 × 10³ PFU), HSV-1 infection was fatal in all of the mice. The LD₅₀ of i.c.-inoculated KOS was approximately 1.5 × 10³ PFU (Table 3). It should be noted that in a separate experiment, 100% of animals died after i.c. inoculation of 1.5 × 10³ PFU KOS (Table 2). At death, infectious virus could be recovered from brain tissue homogenates. The titer of infectious KOS virus recovered after i.c. inoculation was about 3 logs greater than the inoculated dose (3 × 10⁶ PFU after 3 × 10³-PFU inoculation and 5 × 10⁶ PFU after 6 × 10³-PFU inoculation on day 3 or 4 p.i.). The persistence of viral DNA in brain tissue was detected by PCR amplification, using primers from ICP6 (ribonucleotide reductase [RR]), thymidine kinase (TK), and glycoprotein E (gE), which amplify sequences of 221, 273, and 320 bp, respectively (Table 1). In all brains from KOS-inoculated survivors analyzed at 56 and 112 days p.i., viral DNA was detectable (Fig. 3A; Table 4).

Attempts to reactivate KOS by superinfection with G207. We attempted to reactivate i.c. KOS virus or generate neurovirulent recombinants by superinfection with a high dose of G207 (10⁷ PFU). At 2 months after KOS i.c. inoculation, a time when HSV-1 DNA was detectable in the brain by PCR, surviving mice were inoculated with G207 at the same stereotactic coordinates as the prior KOS injection. Some of these animals were followed for over 1 year with no sign of disease, suggesting that neurovirulent recombinants were not generated. The persistence of viral genomes after G207 superinfection was determined by PCR (Table 4). The LacZ primer (Table 1) was used to differentially identify G207 sequences, with the RR, TK, and gE primers common to both G207 and KOS DNA (Fig. 3B). We have been unable to generate useful PCR primers that would uniquely identify KOS DNA and not G207. Amplification of the cellular fatty acid binding protein

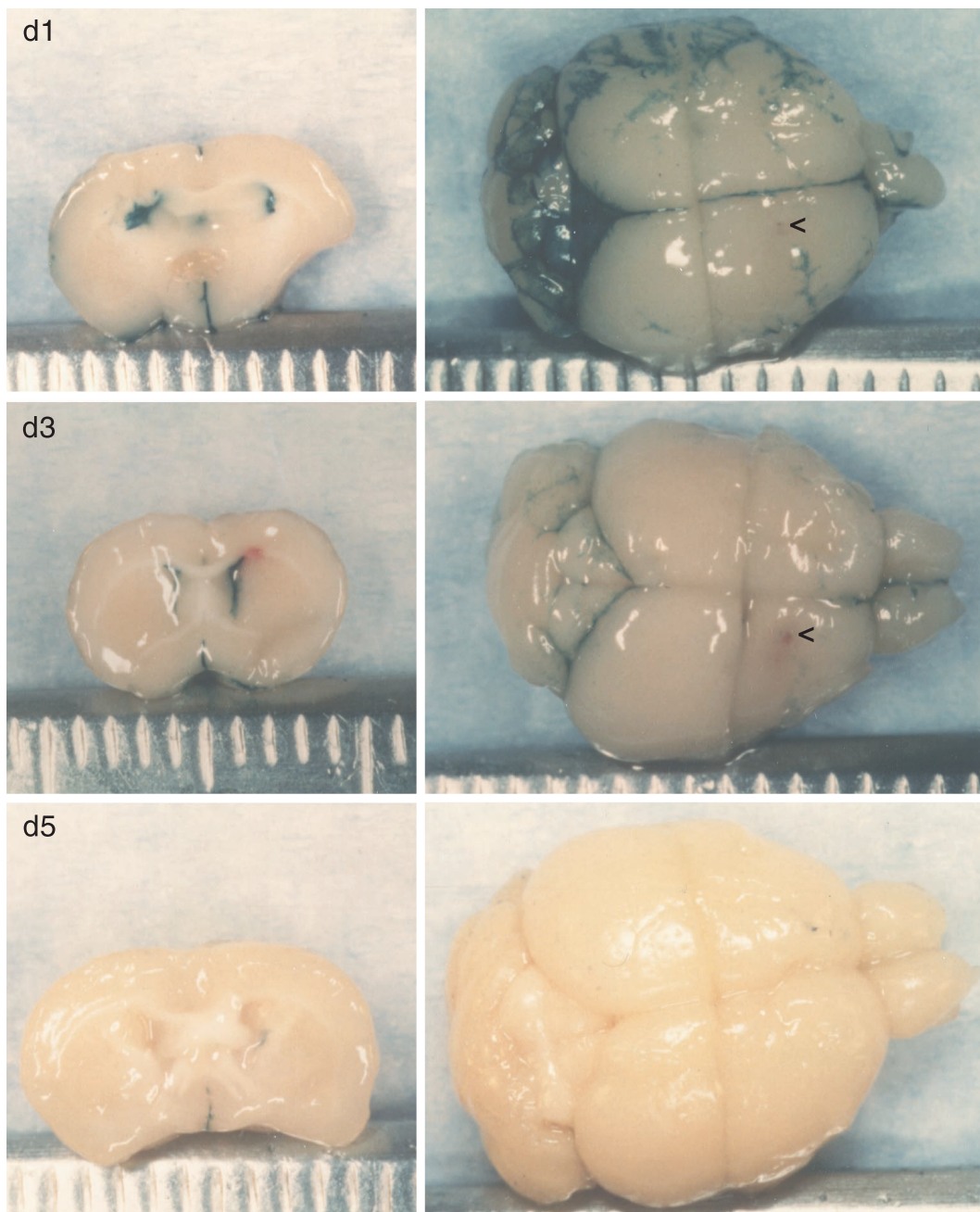


FIG. 1. Intracerebroventricular injection of G207. Brains were isolated 1, 3, and 5 days after i.c.v. injection of G207 (10^7 PFU) and stained for LacZ expression by X-Gal histochemistry. On the left are brains cut coronally through the ventricles; on the right are the upper surface of the brain, with the olfactory bulb to the right and cerebellum to the left. A small red spot is visible at the site of injection (<) in the day 3 (d3) brain below the midline, as well as above the ventricle in the coronal section (left).

gene (Fx) served as internal control and to normalize the amount of tissue DNA in all experiments. G207 and likely KOS DNA sequences were detected from 8 to 24 weeks after the second injection with G207 (Table 4). To determine the sensitivity of the PCR assay, we mixed known amounts of KOS or G207 virus with normal mouse brain tissue and extracted the DNA as was done with the inoculated brain samples. We could detect approximately 10 PFU equivalents of KOS and G207 with the ICP6 and LacZ primers, respectively (Fig. 3C).

It was possible that KOS reactivated but was unable to cause detectable disease. Therefore, we assayed brain homogenates for infectious virus on days 1, 2, 4, 7, 14, and 30 after G207 challenge by plaque assay (Fig. 4). G207 plaques could be distinguished from KOS plaques because of the expression of β -galactosidase which is histochemically stained with X-Gal. Infectious G207 could be detected 1 day after injection (4×10^5 PFU/brain), with titers declining by day 2 and no plaques detectable at day 4 or later (Fig. 4). We were unable to detect

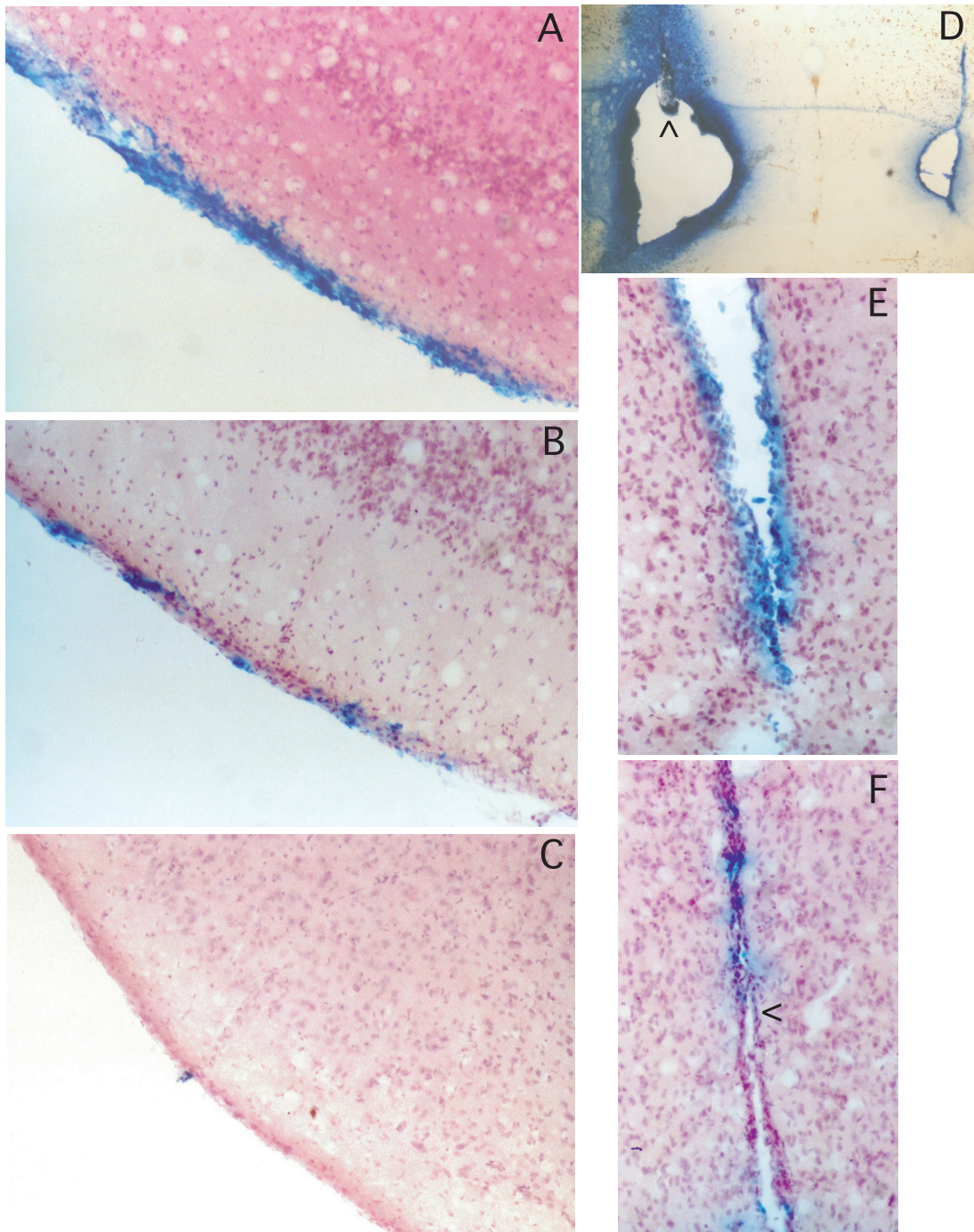


FIG. 2. Histology of i.c.v.-injected brains. Mouse brains were isolated 1 (A), 3 (B and E), or 5 (C and F) days after i.c.v. injection of G207 (10^7 PFU) as in Fig. 1, sectioned, and counterstained. X-Gal staining of meningeal cells is apparent in the subarachnoid space adjacent to the piriform cortex (A to C) and ependymal cells of the third ventricle (E and F). There is no evidence of meningitis or loss of ependymal cells (<, ependymal single-cell layer). The accuracy of the i.c.v. injections was confirmed in separate animals by injection of Evans blue dye (D). Injection site in the ventricle is indicated by an arrowhead; Evans blue dye is apparent in the injected and noninjected, contralateral ventricle.

any KOS plaques (white after X-Gal staining) at any of these time points, in contrast to the results obtained after the initial i.c. inoculation of KOS.

DISCUSSION

G207 is a second-generation, attenuated replication-competent HSV vector that was constructed for the original purpose

of treating brain tumors. We have shown that G207 is efficacious in the treatment of brain tumors, inhibiting the growth and/or prolonging survival in animal models of human glioma (57), meningioma (88), and breast cancer metastatic to the brain (79). G207 is also effective in treating a variety of solid peripheral human tumors, such as head and neck squamous cell carcinoma, breast adenocarcinoma, prostate adenocarci-

TABLE 3. Incidence of mortality after i.c. inoculation^a

Inoculation	Viral dose (PFU, 10 ³)	Time of death (days p.i.)	Mortality (%)
KOS	0.25		0
	0.5	4, 5, 5	37
	0.75	10, 11	25
	1.5	3, 3, 3, 4, 7	58
	2.0	5, 5, 6, 7	50
	3.0	2, 3, 3, 3, 3, 4, 4, 7	100
	6.0	2, 3, 3, 3, 3, 5, 6	100
VB			0

^a Animals were observed from 60 to 240 days p.i. There were 8 animals at each dose of HSV-1 KOS and 12 animals in the control VB group. These animals were different from those described in Table 2. Surviving animals after inoculations of 5×10^2 to 20×10^2 PFU of KOS were used in the G207 challenge studies (Table 4).

noma, and colorectal cancer, after intraneoplastic injection (9, 37, 79, 82). Hepatic portal vein injection of G207 was shown to reduce the number of liver metastases in a syngeneic hepatoma model (37). Recently, we found that i.v. injection of G207 in nude mice was able to induce tumor regression in distant subcutaneous LNCaP and DU-145 human prostate adenocarcinomas (82). In humans, HSV infection of the CNS often causes a rapidly debilitating or fatal encephalitis (13). Blood-borne virus concentrates in the liver and HSV infections of the liver can cause a fulminant hepatitis (22). It is therefore important to demonstrate that inoculation with G207 in the brain and possible leakage to CSF or blood will not pose a significant risk to any patient undergoing therapy.

G207 is derived from HSV-1 strain F, one of the most attenuated laboratory strains (16), and contains engineered mutations in two viral genes affecting neurovirulence, γ 34.5 (10, 77, 78) and ICP6 (8, 28, 87). These features were incorporated into G207 in order to maximally decrease neurovirulence and yet maintain the ability of the virus to replicate in tumor cells. We found no morbidity associated with injection of 10^7 PFU of G207 into the cerebral cortex, ventricles, liver, or tail vein of BALB/c mice. There was no or minimal G207 replication after i.c. inoculation, so that the titer of recoverable infectious virus decreased from the input of 10^7 PFU to 2×10^2 PFU on day 2 p.i. to undetectable on day 4. This recovered G207 could be remaining input virus or newly synthesized. Intracerebral injection of 10^6 PFU R3613, the strain F γ 34.5 Δ parent of G207, was found to cause no deaths, and only 10^2 PFU/g of brain tissue could be recovered (10).

In contrast, other attenuated, replication-competent HSV vectors being used for cancer therapy are derived from the strain 17⁺ background, and γ 34.5 mutations in this background are more neurovirulent than those in the strain F background (35, 47, 52, 54). Intracerebral injection of 10^6 PFU of 1716, γ 34.5 Δ in strain 17⁺, in both AO rats and BALB/c mice, caused a severe inflammatory response in the brain that was accompanied by excessive weight loss and clinical illness (54). Interestingly, C3H/He mice remained healthy after similar injections (54), even though C3H/He mice are similar to BALB/c mice in sensitivity to wild-type HSV infection (44). After i.c. inoculation of R3616 or 1716, virus is widely distributed via retrograde transport in neurons, and there is some expression of viral antigens and possible viral replication within the first week, which then ceases (48, 55). In studies using G207 as a helper virus for defective HSV vectors, we found that inoculation in the rat substantia nigra resulted in LacZ expression only in cells surrounding the site of inoculation and not at

retrograde sites that were identified by expression of the alkaline phosphatase reporter gene on the defective vector (59).

A feature common to both R3616 and 1716 is a predilection to replicate in and destroy ependymal cells in the ventricles. When R3616 was injected into the striatum of mice, virus spread to the ventricles, presumably through leakage into the CSF, so that within 7 days the ependymal cells in the lateral ventricles had disappeared (48). Direct inoculation of 1716 into the ventricles of BALB/c mice also led to a loss of ependymal cells and hydrocephalus but no mortality (34). When 1716 was injected into the ventricles of nude mice, it caused high mortality even at low doses (10^3 PFU) (41). Intrathecal injection of *hrR3* (KOS backbone with same ICP6⁻ mutation as G207) into rats caused significant neurologic morbidity and some mortality (38). In contrast, animals receiving i.c.v. inoculations of G207 exhibited no symptoms of disease. The virus spread throughout the ventricular system, with large numbers of cells bilaterally expressing LacZ within the first day. LacZ expression then rapidly decreased, so that by day 5 postinoculation very few positive cells were detected, with no apparent loss of ependymal cells. LacZ expression is a marker for active viral transcription. In G207, LacZ is driven by the ICP6 promoter, a leaky early (E) or β promoter, that is activated by VP16 (α TIF) and ICP0 but not ICP4 (14, 24, 76).

Mutations in a number of nonessential viral genes, including the γ 34.5 (61, 66, 71, 86), ICP6 (27, 28), TK (12), uracil DNA glycosylase (UL2) (62), and ICP0 (42) genes, affect the ability of HSV to establish latency in the PNS and to be reactivated. In many cases the degree of reactivation and/or establishment of latency is dependent on the in vivo model, animal species, route of infection (e.g., corneal infection/latent trigeminal ganglia, footpad infection/latent dorsal root ganglia, or vaginal infection/latent sacral ganglia) (71), and means of inducing reactivation (e.g., explant cocultivation or in vivo stimuli) (20, 62). Based on the ability of HSV to establish latency, replication-defective mutants of HSV have been used for gene delivery to the CNS, usually by injection into the hippocampal region (4, 21, 64). Unfortunately, in most cases the delivered gene is only transiently expressed. However, viral DNA transcripts and LATs can be detected for long periods of time in the region of injection in the brain (4, 21, 64), suggesting that a latent infection has been established. Intracerebral inoculation of strain 1716 was similarly found to lead to the establishment of a latent infection in large numbers of neurons, as detected by LAT expression (35). We found that G207 DNA was present in the brains of mice 6 months after i.c. inoculation.

With the recent findings of HSV DNA in the CNS of patients dying without encephalitis (2, 43, 68), it became important to determine whether i.c. injection of G207 might lead to reactivation of or recombination with latent virus, leading to a neurovirulent phenotype. Mixed infections in the periphery with two nonneuroinvasive HSV-1 strains can lead to a lethal infection that spreads to the brain at doses logs less than for each of the viruses alone (29). This is due to both complementation and generation of neuroinvasive recombinants (70) and occurs with mixtures of HSV-1 KOS plus F (parental strain of G207) (73). There have been only a few reports of attempts to establish HSV-1 latency models in the CNS, most unable to demonstrate reactivation (35, 45, 72, 75, 85). For example, using a variety of explant protocols with mouse brainstem, Steiner et al. were unable to demonstrate any evidence of reactivation in 44 samples where reactivation from trigeminal ganglia was readily apparent (72). In an HSV-1 latency model established in motor neurons of the spinal cord, explanting spinal cord did not lead to viral reactivation, in contrast to

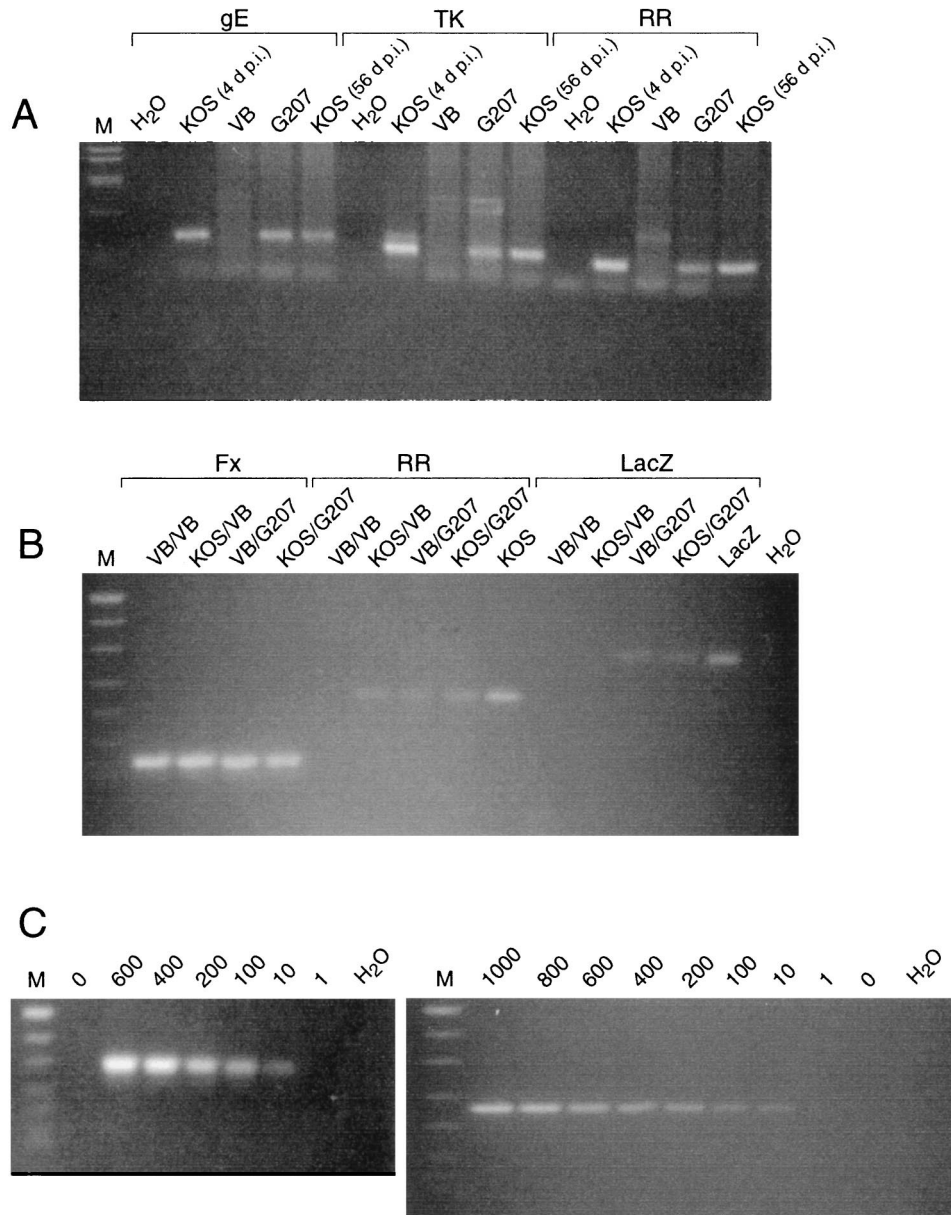


FIG. 3. (A) Persistence of viral DNA in the mouse brain after i.c. inoculation of HSV-1. DNA was isolated from the brains of the following animals: KOS (6×10^3 PFU) at time of death, 4 days p.i. (d p.i.) (lanes 2, 7, and 12); VB at 56 days postinoculation (lanes 3, 8, and 13); G207 (10^7 PFU) at 56 days p.i. (lanes 4, 9, and 14) and KOS (10^3 PFU) survivor at 56 days p.i. [KOS (56 d p.i.)] (lanes 5, 10, and 15). Isolated DNA was amplified with the gE (lanes 1 to 5), TK (lanes 6 to 10), and RR (lanes 11 to 15) primer pairs (Table 1), separated by agarose gel (2.5% NuSieve) electrophoresis, and visualized with ethidium bromide. The controls (lanes 1, 6, and 11) contained water in place of DNA. DNA size markers (M) are as in panel B. (B) Detection of HSV DNA sequences in the brain by PCR. DNA was isolated from the brains of animals listed in Table 4, after injections (first/second) as indicated above the lanes. Isolated DNA was amplified with the Fx (lanes 1 to 4), RR (lanes 5 to 9), and LacZ (lanes 10 to 14) primer pairs (Table 1), separated by agarose gel (2.5% NuSieve) electrophoresis, and visualized with ethidium bromide. The DNA size markers (M) are MW VIII from Boehringer Mannheim (501/489, 404, 320, 242, 190, 147, and 127 bp), the positive control for KOS (lane 9) is 100 PFU equivalents of KOS mixed with mouse brain, the positive control for LacZ (lane 14) is 10 ng of pHCL (31), and the negative control (lane 15) is water in place of DNA. The LacZ primer pair uniquely detects G207 DNA. (C) Detection sensitivity of PCR assay. HSV-1 G207 (left) or KOS (right) was mixed with mouse brain, and DNA was isolated. DNA size markers (M) are as in panel B. G207 DNA (0, 600, 400, 200, 100, 10, and 1 PFU equivalents), and water in place of DNA in lane 8, was amplified with the LacZ primer pair (Table 1) (left); KOS DNA (1,000, 800, 600, 400, 200, 100, 10, 1, and 0 PFU equivalents), and water in place of DNA in lane 10, was amplified with the RR primer pair (Table 1) (right).

100% reactivation from latently infected dorsal root ganglia (85). In the two studies where latent HSV-1 could be reactivated from the mouse CNS, virus was identified in explants of 1 of 20 brain hemispheres and none of 20 brainstems, whereas it could be isolated from 38 of 40 trigeminal ganglia (7), and in 4 of 53 explanted spinal cords, whereas 36 of 53 spinal ganglia

were positive (36). The difficulty in demonstrating HSV reactivation from the CNS as opposed to the PNS may be due to the ability to explant and maintain viable ganglia from the adult PNS, whereas it is not readily possible to maintain viable tissue from the adult brain in vitro.

To establish a mouse model with persistent HSV DNA in

TABLE 4. Detection of viral sequences in the brain by PCR^a

Injection		Primer					No. of animals	
1st	2nd	RR	TK	gE	LacZ	Fx	PCR	Alive
KOS	VB	+	+	+	-	+	1	3
	G207	+	+	+	+	+	3	5
VB	VB	-	-	-	-	+	2	3
	G207	+	+	+	+	+	2	3

^a Three-week-old BALB/c female mice were i.c. inoculated with $\leq 2 \times 10^3$ PFU of KOS or VB (first injection); after viral latency had been established (8 to 9 weeks), G207 (10^7 PFU) or VB was inoculated at the same stereotactic site (second injection). Animals were sacrificed 8 weeks after the second injection, except in the KOS/G207 group, where an animal was sacrificed at 8, 16, and 24 weeks (PCR); DNA was extracted from brain tissue and subjected to PCR with the indicated primers (Table 1). The remainder of the animals were alive and well at 1.7 years.

the brain, we i.c. inoculated mice with approximately 1 LD₅₀ of wild-type HSV-1 KOS 1.1, which was about $10^{3.2}$ PFU. This can be compared to previously determined LD₅₀s of $10^{2.2}$ PFU for KOS 321 in DBA/2 mice (11) and $10^{1.7}$ PFU for KOS-63 in BALB/c mice (16). Large amounts of infectious virus ($>10^6$ PFU) could be isolated from the brains of animals that did not survive KOS i.c. injection. It seems likely that in those animals that survived, significant numbers of cells were infected. Superinfection with a high dose of G207 (10^7 PFU), at the same stereotactic coordinates as the initial inoculation with HSV-1 KOS, did not lead to detectable reactivation of infectious virus, nor did it result in disease through the generation of neurovirulent recombinants. While this work was in progress, Wang et al. (83) similarly used an i.c. inoculation of a sublethal dose of HSV-1 KOS to establish latency in the brains of rats. They were unable to detect ICP6 transcripts from this virus, as a marker of reactivation, after i.c. inoculation of *hrR3* (ICP6⁻) at the same stereotactic coordinates (83).

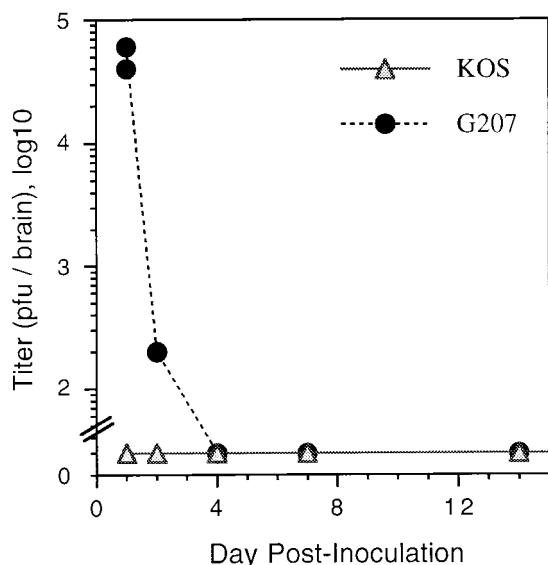


FIG. 4. Recovery of infectious HSV from G207-challenged brains. Whole brain homogenates, from surviving KOS i.c.-inoculated BALB/c mice that were rechallenged with G207 (10^7 PFU) on day 0, were titered on Vero cells, and the PFU per brain was determined. Plaques that stained with X-Gal were considered G207, and those that did not were considered KOS. No plaques were detected from day 4 onward; no white plaques were found at any time point. The minimal detectable titer was >30 PFU per brain. Two animals were sacrificed at each time point.

These studies provide strong support for the safety of G207 when inoculated into the brain, liver, or systemic venous circulation of mice, even at high doses. In light of the serious neurologic complications that HSV infection causes, it is rather remarkable that mutations in two viral genes can have such a dramatic effect on the pathogenesis of the virus. It will be important to determine what aspects of viral replication or virus-host interactions are blocked in the CNS or peripheral organs that permit G207 replication and destruction of brain and other solid tumors but not normal tissue.

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