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ACQUIRED DISEASES ***RESEARCH ARTICLE***

Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial

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G207 is a conditionally replicating derivative of herpes simplex virus (HSV) type-1 strain F engineered with deletions of both $\gamma_1$34.5 loci and a lacZ insertion disabling the U_L39 gene. We have demonstrated the efficacy of G207 in treating malignant glial tumors in athymic mice, as well as the safety of intracerebral G207 inoculation in mice and in Aotus nancymai. We sought to determine the safety of G207 inoculation into cerebral malignant glial tumors in humans. Criteria for inclusion into this dose-escalation study were the diagnosis of histologically proven malignant glioma, Karnofsky score ⩾70, recurrence despite surgery and radiation therapy, and an enhancing lesion greater than 1 cm in diameter. Serial magnetic resonance images were obtained for volumetric analysis. The trial commenced at a dose of 10^6 plaque forming units (p.f.u.) inoculated at a single enhancing site and was completed when the 21st patient was inoculated with 3×10^9 p.f.u. at five sites. While adverse events were noted in some patients, no toxicity or serious adverse events could unequivocally be ascribed to G207. No patient developed HSV encephalitis. We found radiographic and neuropathologic evidence suggestive of anti-tumor activity and long-term presence of viral DNA in some cases. Gene Therapy (2000) **7,** 867–874.

Keywords: *HSV; glioblastoma; anaplastic astrocytoma; human; gene therapy*

Introduction

Malignant gliomas are the most common primary malignant brain tumors, and are almost universally fatal despite aggressive therapies including surgery, radiotherapy and chemotherapy. Patients with glioblastoma multiforme (GBM), ie WHO grade IV astrocytoma, have a median survival of 12–18 months from initial diagnosis and 6–9 months after recurrence.[1,2] Patients with anaplastic astrocytomas (AA), ie WHO grade III lesions, live longer with a median survival of 36–40 months after initial diagnosis and 12–18 months after recurrence.[3,4] Standard management of these tumors includes biopsy and/or tumor resection, followed by external beam radiotherapy, with treatment doses of approximately 6000 cGy. Partial responses to chemotherapy are seen in approximately 30% of tumors, but no significant change in mortality has been shown with this treatment.[1] Due to the lack of success with these standard treatments, recent efforts to improve survival have included various biologic therapies including monoclonal antibodies, immunotherapy and gene therapy.

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We have concentrated our efforts on the development of genetically engineered herpes simplex virus type 1 (HSV-1) for the treatment of malignant glioma. HSV-1 is known to be able to grow in neural tissue and we hypothesized and demonstrated that HSV-1 could grow within and kill tumors derived from nervous system cells.[5] However, wild-type HSV-1 can cause a fatal, hemorrhagic, necrotizing encephalitis in humans. Our studies led to the development of G207, a conditionally replicating HSV-1. G207 contains mutations in both copies of the virus' diploid $\gamma_1$34.5 gene, as well as a disabling *lacZ* insertion in U_L39, the gene encoding the large subunit of the viral ribonucleotide reductase.[6] As a result of these mutations, the intracerebral inoculation of G207 into HSV-sensitive murine and simian primates is not pathogenic.[6–8] These models mimic the most susceptible human populations. Despite these mutations, G207 retains anti-tumor efficacy in a wide variety of *in vitro* and *in vivo* gliomas[6] and other tumor models.[9–11] The combination of promising safety and efficacy profiles led us to explore G207 as a therapeutic agent for malignant gliomas in a phase I, dose escalation study designed to determine the safety of stereotactic inoculation of this genetically engineered HSV-1 for the treatment of recurrent malignant glioma.

Results

Patient characteristics

Twenty-one patients were enrolled in the study between February 1998 and May 1999 (Table 1). The mean age was 54.1 years (range, 38–72). Fifteen men and six women were treated. Sixteen patients entered the study with a diagnosis of GBM. The mean age of GBM patients at diagnosis was 56.1 years (range, 38–72). Patient No. 3 originally was diagnosed with a GBM although a subsequent resection yielded tissue consistent with a gliosarcoma. This patient is included in the GBM group below. There were four patients with anaplastic astrocytoma and one with an anaplastic mixed glioma, hereafter grouped with the AA patients. The mean age of AA patients at diagnosis was 48 years (range, 46–54). Ten tumors were located primarily in the right hemisphere, 10 primarily in the left hemisphere, and one was characterized as bilateral. Six tumors predominantly involved one frontal lobe, five were parietal, three temporal, and one occipital; the remaining six had multilobar tumors. Seventeen patients had undergone craniotomy for debulking and four had undergone stereotactic biopsy without debulking before inoculation. All patients had undergone previous external beam radiotherapy with a minimum biologic effective dose of 5000 cGy. Ten patients had undergone chemotherapy with one or more agents.

The date of original glioma diagnosis preceded G207 treatment by a mean of 13.9 months (range, 2–65). In GBM patients, the diagnosis date preceded G207 treatment by a mean of 9.4 months (range, 4–15), while in AA patients, the diagnosis date preceded G207 treatment by a mean of 28.0 months (range, 2–65).

Table 1 Demographics

Patient No.	*Dose (p.f.u.)*	*Age (years)/ Gender*	*Pathology*	*Location*	*Time from diagnosis to inoculation (months)*	*Time from inoculation to death (last follow-up) (months)*
1	1×10^6	54 m	AA	LP	65	6
2	1×10^6	46 m	AA	BF	20	13
3	1×10^6	54 m	GBM	LP	4	12
4	1×10^7	60 f	GBM	LF	12	10
5	1×10^7	46 f	AA	L T-P	2	(19)
6	1×10^7	50 m	GBM	RT	8	6
7	3×10^7	62 m	GBM	R F-T	12	5
8	3×10^7	52 f	GBM	RF	9	(17)
9	3×10^7	72 f	GBM	LF	12	5
10	1×10^8	60 m	GBM	RF	11	5
11	1×10^8	57 m	GBM	RT	11	3
12	1×10^8	63 f	GBM	LP	6	7
13	3×10^8	69 m	GBM	LT	10	2
14	3×10^8	48 f	AA	L F-P	33	1
15	3×10^8	38 m	GBM	RO	9	6
16	1×10^9	60 m	GBM	LP	6	11
17	1×10^9	56 f	GBM	LF	5	(8)
18	1×10^9	46 m	AA	R P-T	20	4
19	3×10^9	43 m	GBM	R F-T	12	(7)
20	3×10^9	51 m	GBM	RF	15	5
21	3×10^9	50 m	GBM	R F-T	9	5

L, left; R, right; F, frontal; T, temporal; P, parietal; O, occipital.

Neurological status

The mean mini-mental status examination (MMSE) score pre-inoculation was 28.4 (s.d. 2.0). The mean MMSE score at day 4 was 27.4 (s.d. 5.1) and at 1 month was 27.4 (s.d. 5.48). The mean MMSE score was 27.5 (s.d. 5.6) in the 12 patients remaining in the study at 3 months.

The mean Karnofsky score pre-inoculation was 84.3 (s.d. 12.5) and at 1 month was 83.8 (s.d. 13.6). The mean Karnofsky score was 81.7 (s.d. 18.5) in the 12 patients remaining in the study at 3 months. An improvement in Karnofsky score was observed in six of 21 (29%) patients at some time after inoculation.

Antibody status and conversion

Before inoculation, 14 of 19 patients were positive for HSV-1 antibody and five were negative. Two patients (Nos 14 and 19) did not have the presence or absence of serum anti-HSV-1 antibodies determined before entry into the protocol (Table 1). Patient No. 21, who was injected at the highest dose level, was the only HSV-1 seronegative patient who became seropositive after G207 treatment.

Patient No. 12 had a saliva culture positive for HSV-1 at the 3 month follow-up visit. X-gal staining of this sample was negative for *lacZ* expression. Southern blot hybridization was carried out on this sample using three enzymes and demonstrated that γ 34.5 and ICP6 were present. Also, the cultured virus contained various restriction site polymorphisms that were not present in HSV-1, strain (F), the parent virus of G207 (Neurovir Therapeutics Inc). These findings indicate that the HSV-1 recovered from the saliva culture did not originate from the G207 inoculation. All other HSV cultures were negative.

MRI tumor volumetrics

MRI evaluations were performed using an eigenvalue algorithm to minimize the introduction of interobserver variability or other bias.[12] This algorithm measured enhancing tumor volumes only, which was thought to represent the viable portion of the tumors. A single patient had a pacemaker and underwent CT scans and volumetric MRI data could not be performed. The mean enhancement volume was 39 cc before G207 inoculation, 43 cc at 4 days, 55 cc at 1 month, 64 cc at 3 months, 26 cc at 6 months (Table 2). Six of 20 patients had a decrease in their enhancement volume between the preoperation scan and the 1 month post-inoculation MRI. Eight of 20 patients had a decrease in their enhancement volume between the 4 day scan and the 1 month post-inoculation scan (Figure 1). Due to the variability in the time period between the pre-inoculation scan and the inoculation date, significant growth occurred in some tumors before inoculation. Therefore, the 4 day post-inoculation MRI is

Table 2 MRI volumetric data

MRI	*Number*	*Mean (cc)*	*Range (cc)*
Pre-inoculation	20	39	3–90
4 day	20	43	4–118
1 month	20	55	3–154
3 month	13	64	2–249
6 month	3	26	1–61

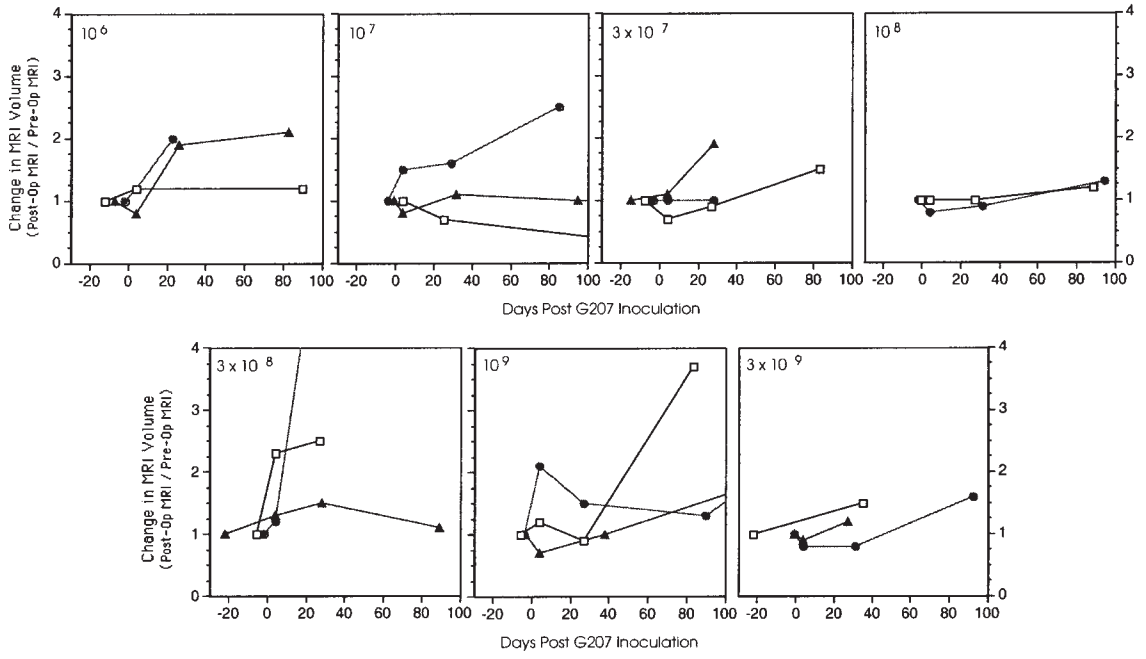

Figure 1 *Individual patients' MRI volumes are grouped by cohort. The x-axis is the number of days from injection. The y-axis is the growth ratio as defined by the enhancing tissue volume, after inoculation divided by pre-inoculation. Volumes were calculated using an eigenvalue algorithm as described in the text.*

probably the best baseline for assessing any changes in enhancement that might be due to G207.

Patient No. 18 had a 25% decrease in enhancing mass volume between the 4 day (80 cc) and 1 month (61 cc) MRI. Patients 3 and 4 had prolonged decreases in enhancement volume at the site of inoculation lasting 5–9 months. Patient No. 3 developed growth of a satellite lesion approximately 2–3 cm from the initial inoculation site. This lesion was treated with a second inoculation 11 months after the first inoculation. The satellite lesion was histologically a gliosarcoma at autopsy. Patient No. 4 is described in more detail below.

Progression and survival

As of the time of submission four patients (AA 1, GBM 3) remain alive a mean of 12.8 months (range, 7–19) following inoculation. Mean time from inoculation to progression was 3.5 months (range, 0–20; $n = 21$). Excluding surviving patients, mean time from inoculation to death was 6.2 months (range, 1–13, $n = 17$). Mean survival from date of diagnosis for GBM patients was 15.9 months (range, 12–22, $n = 13$). Mean survival from date of diagnosis for AA patients was 40.5 months (range, 24–71, $n = 4$). After G207 inoculation and subsequent progression of tumor, four patients received chemotherapy and five patients underwent surgical debulking. Patient No. 6 developed a liver metastasis of his GBM. No other patient developed a clinically or autopsy-detected metastasis.

Adverse events

Patients 4, 12 and 14 underwent post-inoculation stereotactic biopsy to investigate clinical deterioration.

Patient 4's tumor (1×10^7 p.f.u.) was histologically a GBM. Following G207 inoculation MRI showed a progressive decrease in the enhancing mass with decreased mass effect. There was no evidence of increased T2 signal to suggest inflammation (Figure 2). She was driving and living independently with a Karnofsky score of 100. Nine months after inoculation she presented with an acute loss of consciousness. Stereotactic biopsy showed only changes consistent with necrosis. She remained comatose for approximately 1 month before expiring. The autopsy revealed pneumonia and subacute infarctions of both middle and left anterior cerebral artery territories. She had no evidence of residual tumor or inflammation and HSV immunostaining was negative.

Patient 12's tumor (1×10^8 p.f.u.) was histologically a GBM. She was stable following G207 inoculation, but approximately 3 months later demonstrated a deteriorating Karnofsky score, new focal deficits, and a progressive decrease in mental status. This deterioration was not explained by tumor progression on MRI. Stereotactic biopsy revealed histologic evidence of tumor that was adjacent to normal white matter. No evidence of HSV was seen by immunostaining, and there was no evidence of new inflammatory changes when compared with her previous resection specimen. She continued to deteriorate, and died 7.5 months after treatment. She had previously participated in another phase I trial examining a radiation sensitizing agent. Her family refused further imaging and autopsy.

Patient 14's tumor (3×10^8 p.f.u.) was histologically an AA. She developed an acute change in mental status and dysphasia within 24 h of inoculation, but developed neither fever nor an abnormal elevation in white blood cell count. Repeat MRI scan performed 1 day after inoculation demonstrated progression of her enhancing mass, but no evidence of edema or hemorrhage. A minimal amount of hemorrhage was seen in the region of the needle track as well as increased enhancement on an MRI done 8 days after inoculation, but no change in the

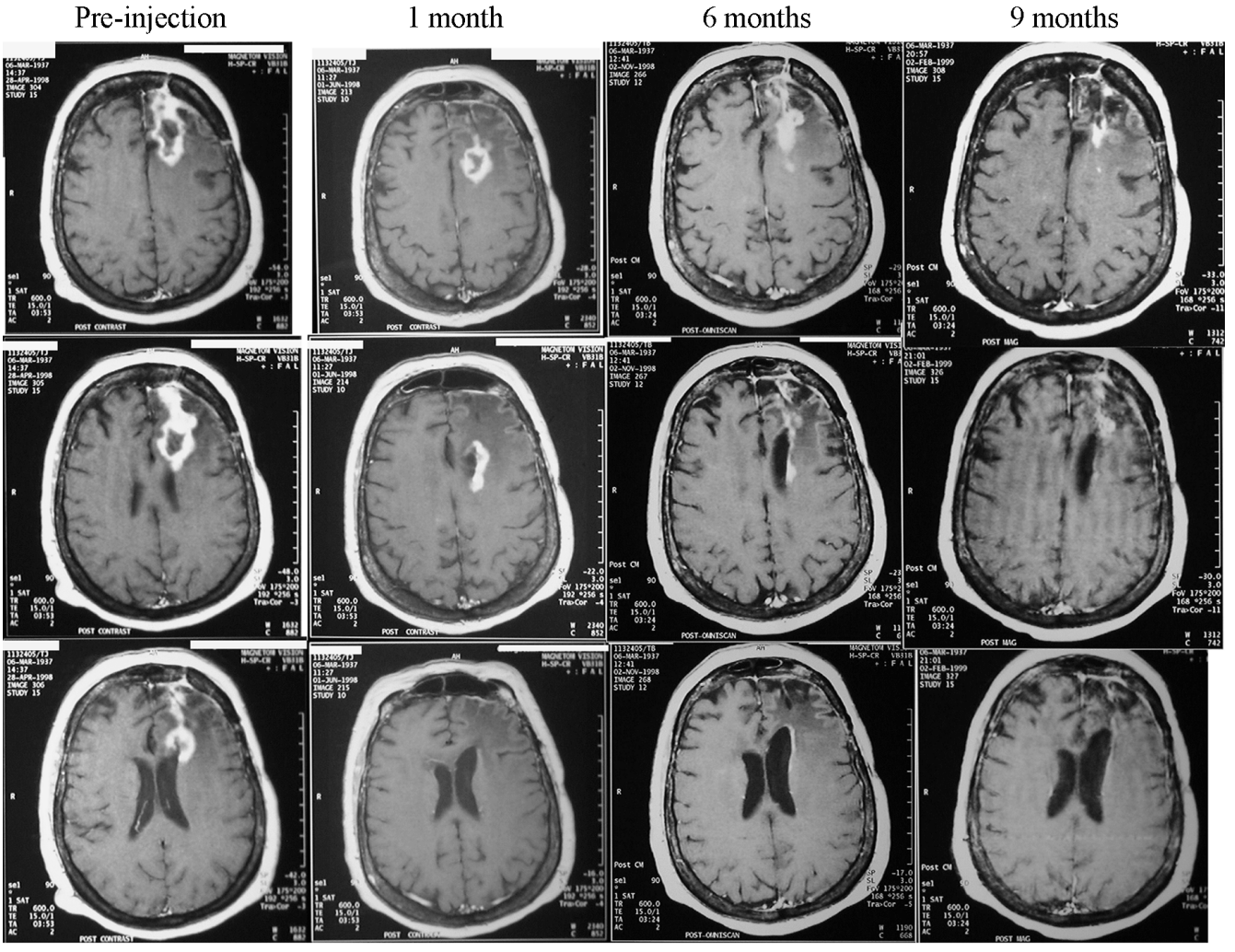

Figure 2 *Patient No. 4 MRIs showing regression. Patient No. 4 T1-weighted gadolinium enhanced MRI images. Volumes pre-injected: 21.5 cc; 1 month 23.4 cc; 6 months 21.1 cc; 9 months 17.1 cc.*

amount of edema. The increase in enhancement (from 50 cc to 117 cc) appeared to occur primarily in the previously nonenhancing portion of the tumor and not in the surrounding brain parenchyma. A stereotactic biopsy was performed 14 days after inoculation to verify that no encephalitis had developed. Three biopsy specimens were taken; one in the region of G207 inoculation, as well as 1 and 2 cm distant. These demonstrated viable tumor with increased cellularity compared with her pre-treatment biopsy, consistent with tumor progression. There was no evidence of inflammatory changes or other findings to suggest encephalitis. Immunostaining of all three specimens for HSV was negative, as was viral culture for HSV. PCR was negative for *lacZ* sequence and positive for HSV-1 sequences *pol* and gB. Inadequate sample remained for retesting this result. The patient improved with dexamethasone administration and became ambulatory but remained confused and somewhat dysphasic. She died 37 days after G207 inoculation, but no autopsy was obtained.

Patients 6 and 19 had temporal lobe masses injected and both required extensions of their post-inoculation hospital stays. Patient 6 had mild increased weakness and tumor progression seen on MRI scan. His increased weakness responded promptly to increased dexamethasone administration. Patient 19 developed a slowed affect within 12 h of inoculation and a CT scan at 24 h demonstrated punctate hemorrhages associated with the five inoculation sites. He responded to increased dexamethasone and regained his pre-operative level of function within 2 days.

Tissue analysis

Biopsy specimens from six patients were analyzed after inoculation. Three specimens were from the post-inoculation diagnostic biopsies described above. Specimens from four patients (Nos 1, 3, 7 and 8) were from re-resections performed 60, 157, 56 and 97 (mean 93) days after inoculation, respectively.

Patients 3 and 8 had specimens that were positive for both HSV-1 and *lacZ* sequence by PCR, indicating that G207 DNA was present in the specimen. These two positive specimens were from resections done 157 and 56 days after inoculation at dose levels of 1×10^6 and 3×10^7 p.f.u.

The brains of five patients (Nos 2, 3, 4, 11 and 20) were

available for analysis at autopsy (four were formalin fixed, the fifth was frozen at −80°C). No evidence of encephalitis, white matter toxicity, or inflammatory changes was present. HSV-1 immunostaining was negative. Patient No. 2 had a bifrontal, 'butterfly' glioma. Histological examination of three of these five brains revealed that their tumors remained localized to the left internal capsule and thalamus (No. 3), right temporal lobe (No. 11), and right frontal lobe (No. 20). Patient No. 20 was diagnosed at the age of 50 and was injected 14 months after his original diagnosis of a GBM. Progression of disease led to an autopsy 5 months after inoculation. His right frontal tumor showed the necrosis and pseudopallisading typical of a GBM. There was no evidence of infiltration into or across the corpus callosum in four of these five patients. Patient No. 4 died due to a stroke 9 months after inoculation and had no evidence of GBM on serial sections of the brain.

No specimen from any patients had evidence of encephalitis on routine hematoxylin and eosin staining, and no HSV-1 antigen was detected by immunostaining.

Discussion

We report the results of the first North American human trial of a herpes simplex virus specifically engineered for the treatment of intracerebral malignancy.[13] We have shown that doses up to 3×10^9 p.f.u. of this conditionally replicating virus can be inoculated safely into brain tumors without the development of encephalitis. No patients developed MRI, laboratory or pathologic evidence of encephalitis. We are still following our four surviving patients and have not documented any evidence of long-term toxicity from this treatment. While some patients developed complications frequently seen with malignant glioma, including death, none of these complications or deaths could be unequivocally ascribed to G207.

However, it is not always possible to know the cause of adverse events in this group of patients. Two patients from our study merit specific discussion. Patient No. 6 developed a liver metastasis, which was first identified after treatment with G207. While rare, extraneural metastases are seen with GBM in 0.1–0.5% of cases, usually in the lung, lymph nodes, bone and liver.[14,15] While the possibility that this was related to G207 therapy cannot be excluded, no other metastatic lesions were found in our trial.

A second patient, No. 14, developed mental status changes and dysphasia which could have been due to surgical trauma, tumor edema, or viral toxicity. However, fever was not present. MRI showed an increase in enhancement, which appeared to correspond to tumorous regions that had not enhanced before G207 inoculation. No increase in the amount of hyperintense T2 signal was appreciated after inoculation. Although biopsy specimens taken from three sites did not show encephalitis or HSV antigen, and cultures were negative for HSV from all three sites, stereotactic biopsies have limitations. Unfortunately, no autopsy was obtained, so a definitive determination of the reason for her decline cannot be made. While the possibility of G207-related toxicity cannot be excluded, six patients were treated at higher dose levels (including one AA patient) without any similar events.

The HSV-1 antibody status of our patients mirrored that of the general population.[16] Of the five patients negative for HSV-1 antibody before inoculation, patient No. 21 injected with 3×10^9 p.f.u., seroconverted. This suggests that despite the known immunosuppressive effects of malignant glioma and chronic dexamethasone treatment, an immune response to HSV-1 can be mounted by at least some fraction of malignant glioma patients after treatment at the highest dose levels of G207.

Neuropathologic evaluation was performed on five autopsied brains after G207 treatment. One GBM patient who had been treated previously with surgery and radiation had no evidence of residual or recurrent glioma at autopsy. The tumors of three other patients remained localized to a single geographic region. This finding is very atypical in malignant gliomas, which by microscopic examination almost always extend (often by single cells seen scattered through sections or in subpial locations) by infiltration into the underlying white matter, generally crossing into the contralateral hemisphere via the corpus callosum.

Malignant glioma was chosen for study due to its lack of response to traditional therapeutics and near uniform lethality. Ninety percent of gliomas recur locally, within 2 cm of their resection margin, and systemic metastases are rare. As a result, these brain tumors are excellent targets for intervention using conditionally replicating viruses such as HSV-1 which replicate and kill tumor cells, while sparing normal nervous tissue.

Currently, many centers are investigating a variety of vectors for use in treating malignant gliomas. The vectors most widely studied to date have been retrovirus and adenovirus. Retroviruses are difficult to produce at high titers, require packaging cell lines, have significant limits to genetic insert size, have a potential for insertional mutagenesis and do not transduce non-dividing cells, including quiescent tumor cells.[17] Adenoviruses do not require a producer cell line and can generate relatively high levels of foreign gene product, but this is usually due to transient gene expression, and may be associated with an overwhelming inflammatory response. Furthermore, adenoviruses are promiscuous in their host cell range, and are also limited in the size of genetic insert that may be utilized. Most adenovirus vectors now in clinical trials are non-replicating.[17] Recently, a replicating adenovirus (Onyx-0115) has been tested in head and neck cancers and has entered testing in human glioma therapy.

HSV-1 is a DNA virus with a well-studied genome. Approximately 90% of the adult population has acquired antibodies due to previous exposure to the virus.[18] While neurovirulent in its wild-type form, a variety of mutations can be introduced which abrogate this toxicity. The neurovirulence gene, $\gamma_1$34.5, is present in two copies in the wild-type virus. Both copies can be deleted while allowing the virus to maintain its anti-tumor effects.[19] Additionally, HSV-1 requires the enzyme ribonucleotide reductase for replication. Viruses defective in ribonucleotide reductase can still replicate in rapidly dividing cells by presumably using the cellular ribonucleotide reductase provided *in trans*. Such viruses cannot, however, replicate and lyse post-mitotic cells such as neurons and most other cells making up the vast majority of normal adult brain.[17,20]

A major and unique advantage of HSV-1 as a vector for treating gliomas and other malignancies is the avail-

ability of effective anti-viral medications already in clinical use (eg acyclovir, ganciclovir), which could be utilized if excessive toxicity resulted from treatment with the vector. G207 contains deletions in both copies of the virus' diploid $\gamma_1$34.5 gene and a *lacZ* insertion disabling the large subunit of the viral ribonucleotide reductase.[6] As a result of the ribonucleotide reductase mutation, G207 is hypersensitive to these antiviral compounds when compared with wild-type HSV-1.[6] G207 has been studied in a variety of rodent and primate models designed to determine both its efficacy as an antiglioma agent as well as its safety.[6–8] Antitumor effects and increased survival have been seen in immunocompromised mice with human xenografts, as well as in other murine tumor models in immunocompetent animals. It is evident that the oncolytic effect of the virus accounts for at least a portion of its anti-tumor effects; other studies suggest that an anti-tumor immune response can also be induced by the virus in animal models.[10,11,21]

Safety of G207 has been demonstrated by intracerebral inoculation in naive animals including sensitive mouse strains (BALB/c, A/J strain) and highly susceptible primates (*Aotus nancymai*). No evidence of encephalitis, either clinically or histologically, has been seen in these animals with G207 doses up to 10^9 p.f.u. In contrast, intracerebral inoculation of wild-type virus results in lethality in both animal models at doses of 10^3 p.f.u. Long-term evaluation of *Aotus* has shown no clinical evidence of G207-related toxicity for as long as 3 years after inoculation. Further *Aotus* studies confirmed that G207 was not detectable in other organs via direct culture or at autopsy.[7]

Our phase I study necessarily has some limitations. First, to minimize surgically-induced morbidity which could confound the interpretation of results, a biopsy was not performed before treatment. As a result, some or all of the enhancing mass in some patients could be radiation necrosis rather than viable tumor. We elected not to perform a pre-inoculation biopsy or resection because post-surgical MRI changes may have masked the MRI changes of subclinical viral infection. We were initially concerned that pre-inoculation surgery would have caused gliosis, inflammation and vascular repair, providing proliferating cells that could support productive infection of non-neoplastic tissue.

Second, we were not able to prove *in vivo* replication of the virus due to ethical concerns over inoculation immediately preceding operation for resection. All of our resection specimens were obtained following disease progression, and thus were not obtained within a time-frame during which we would expect to see unequivocal evidence of G207 replication.

Third, to minimize potential surgical complications that might have interfered with evaluation of G207 safety, single site inoculations of G207 in minimal volumes (0.1–0.3 cc) were performed, except at the highest dose level (0.2 cc in each of five sites) where volume requirements necessitated multiple site inoculations. Limited viral distribution within the tumor may have interfered with efficacy.

Our study suggests that G207 can be safely inoculated into human brain tumors at doses up to 3×10^9 p.f.u. Further studies with this agent in the treatment of human glioma are warranted.

Table 3 Dose escalation schedule

No. patients	*Dose (p.f.u.)*	*Volume per locus (cc)*	*No. loci*
3	10^6	0.1	1
3	10^7	0.1	1
3	3×10^7	0.1	1
3	10^8	0.1	1
3	3×10^8	0.1	1
3	10^9	0.3	1
3	3×10^9	0.2	5

Materials and methods

Trial design

A phase I dose escalation design was employed using a modified halflog incremental scheme (Table 3). An initial dosage level of 10^6 p.f.u. or active HSV-1 particles, was chosen based upon preclinical efficacy studies in mice and safety data obtained in both mice and primate studies.[7,8] At each dose level, patients were entered in cohorts of three, with a 10-day waiting period before inoculation of the next patient within each cohort. After accrual to each cohort was completed, we waited 28 days to observe for potential acute toxicities before proceeding with dose escalation. These waiting periods allowed assessment of possible acute toxicity before proceeding with the next inoculation. This protocol and its amendments were approved by the Institutional Review Boards of both Georgetown University Medical Center and the University of Alabama at Birmingham, and reviewed by the Recombinant DNA Advisory Committee of the National Institutes of Health and the Food and Drug Administration.[22]

Inclusion and exclusion criteria

Patients included in the trial had MRI or CT evidence of recurrent or progressive malignant glioma despite standard therapy (Table 4). Standard therapy was defined as

Table 4 Enrollment criteria

- Biopsy-proven glioblastoma multiforme, anaplastic astrocytoma or gliosarcoma.
- One centimeter of enhancing tissue that would not require transgression of the brainstem, basal ganglia or ventricle during a stereotactic inoculation.
- Failed radiation therapy (≥5000 cGy) more than 4 weeks before inoculation.
- Failed surgery more than 4 weeks before inoculation.
- No chemotherapy within 6 weeks of inoculation.
- Karnofsky score greater than or equal to 70.
- Willing to practice birth control.
- No pregnant or lactating females.
- No history of encephalitis, multiple sclerosis or other CNS infection.
- HIV seronegative.
- Tumor growth on MRI following the last treatment undertaken.
- Normal hematologic, hepatic and renal function.
- No prior participation in a viral therapy protocol.
- No active aphthous ulcers.
- No change in steroid dose within 2 weeks of the inoculation.
- No current treatment with any medication active against HSV (eg acyclovir).
- 18 years of age and able to give informed consent.

surgery and/or biopsy followed by external beam radiotherapy (≥5000 cGy biologic effective dose). Some patients received chemotherapy before enrollment, but no chemotherapy could be given within 6 weeks of treatment with G207 inoculation to prevent confounding of trial results. Additionally, patients could not have received debulking surgery within 4 weeks of inoculation to minimize the possibility of G207 viral infection of cells active in the normal reparative response. All patients had histopathological confirmation of their diagnosis by central review.

Patients underwent screening with a history, physical examination, chemistry profile, complete blood count, urinalysis, HIV serology, HSV-1 and HSV-2 antibody titers, HSV-1 cultures of saliva and blood, serum beta-HCG, electrocardiogram, chest radiograph, and volumetric magnetic resonance imaging (MRI). Screening volumetric MRI included axial imaging: fast spin echo, FLAIR, and T1 (pre- and post-gadolinium). T1-weighted images in the coronal and sagittal planes were also included. The image matrix was 256×192 and the field of view was 200 mm. Scan thickness was 10 mm with interscan spacing of 2 mm. Laboratory studies were performed by Covance Laboratories (Indianapolis, IN, USA) unless otherwise specified.

The date of progression was defined as the time at which there was clinical progression, increased steroid dose dependence or any increase in enhancement on imaging. Toxicities were rated using the classification developed by the National Institutes of Health.[23]

Virus handling and operative procedure

Production of G207 following current Good Manufacturing Practices (cGMP) was performed under contract at BioReliance Corporation (formerly Magenta, Rockville, MD, USA), using a process developed at NeuroVir Therapeutics Inc (Vancouver, BC, Canada). The upstream process was roller bottle based and utilized a freeze–thaw step to release virus from cells. Major purification and concentration steps in the downstream process were achieved using size exclusion chromatography and ultracentrifugation, respectively. This process yielded approximately 3×10^{10} p.f.u. final product per 100 roller bottle run with a specific activity of $\geq 5 \times 10^8$ p.f.u./mg. The virus was then stored in 1.0 ml cryovials containing 0.12 ml of G207 suspended in the storage buffer D-PBS/10% glycerin at −60°C. Immediately before surgery, the cryovial with virus was removed from storage and thawed in a bath at 37°C, then centrifuged for 10 s. An aliquot was diluted with sterile saline for injection (USP) to the concentration appropriate for each dose cohort, then transported to the operating room on ice. All handling of the virus and materials potentially contaminated with virus was conducted in accordance with Biosafety Level 2 precautions.

On the morning of surgery, the patients underwent Cosman-Roberts-Wells stereotactic head frame application under local anesthesia, followed by a contrast-enhanced CT scan and target localization. All targets were chosen by the neurosurgeon/investigator at each study site, with the goal of injecting virus in the enhancing (probably actively growing) regions and avoiding inoculation of central non-enhancing (probably necrotic) regions of tumor.

The patients were then taken to the operating room where the virus was stereotactically injected. Needles with stylets (0.7 mm diameter, 220 mm length, Radionics, Burlington, MA, USA) were used for each inoculation. The volume of each needle, as well as the volume of the dead space present during each inoculation, was calculated pre-operatively to allow for precise dosing. Target points were calculated in a standard fashion. A slow, deliberate administration schedule was adopted to avoid reflux of virus into the needle and along the needle track. The needle was passed to the target, the stylet was removed, and the virus was injected slowly over a 2-min interval. Following inoculation, the syringe and needle were left in place for 2 min to allow interstitial diffusion of the inoculum. The stylet was then reinserted over 2 min. Finally, the needle was withdrawn over 2 min. Patients in the final cohort underwent five inoculations according to the same procedure. These five loci were selected by the surgeon to include the superior and inferior poles, as well as three additional equatorial locations of enhancement. No significant egress of the viral preparation was noted along the needle tract during surgery.

The patients were observed in the hospital for 4 days, and a MRI scan was obtained before discharge. Encephalitis was considered if a fever greater than 38°C was present for greater than 48 h, deterioration in neurological status occurred, or there was progressive hemorrhage and/or swelling inside or around the inoculated enhancing tissue. When deemed appropriate by the surgeon, stereotactic biopsies were performed, and examined for histologic and immunohistochemical evidence of HSV encephalitis. Polymerase chain reaction (PCR) was performed to look for evidence of HSV and *lacZ* staining when appropriate. Empiric intravenous acyclovir administration was planned for patients whose clinical pattern was suspicious for HSV encephalitis.

Patients were evaluated with history, physical examination, HSV antibody titer, saliva and blood HSV cultures, as well as MRI pre-operatively and at 4 days, 1 month, 3 months, 6 months and 1 year following inoculation. Neurological examination included mini-mental status testing, Karnofsky grading, and neurological assessment. Patients had sera and saliva cultured at each follow-up visit for evidence of HSV shedding. Follow-up MRI's were classified as complete response, partial response, stable disease or progressive disease. Patients who showed signs of clinical or MRI disease progression were declared a treatment failure and cleared to pursue additional therapy. Even after disease progression, patients were included in clinical, MRI and autopsy follow-up whenever possible.

Note added in proof

Since acceptance of our manuscript, we have been notified of the death of patients 17 and 19, 9 and 10 months following injection.

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