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PRECLINICAL EVALUATION OF A GENETICALLY ENGINEERED HERPES SIMPLEX VIRUS EXPRESSING IL-12

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Abbreviations: GBM, glioblastoma multiforme; HSV, herpes simplex virus; PKR, protein kinase R; ICP, infected cell protein; IL, interleukin; egr-1, early-growth response-1; MEM, minimum essential medium; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; NHP, nonhuman primate; UAB, University of Alabama at Birmingham; USDA, United States Department of Agriculture; MRI, magnetic resonance imaging; FLAIR, fluid attenuated inversion recovery; IHC, immunohistochemistry; PHA, phytohemagglutinin; MST, median survival time.

ABSTRACT

Herpes simplex virus, type 1 (HSV-1) mutants that lack the $\gamma_1$34.5 gene are unable to replicate in the central nervous system but maintain replication competence in dividing cell populations, such as those found in brain tumors. We have previously demonstrated that a $\gamma_1$34.5-deleted HSV-1 expressing murine interleukin-12 (M002) prolonged survival of immunocompetent mice in intracranial models of brain tumors. We hypothesized that M002 would be suitable for use in clinical trials for patients with malignant glioma. To test this hypothesis, we: 1) compared the efficacy of M002 to three other HSV-1 mutants, R3659, R8306 and G207, in murine models of brain tumors; 2) examined the safety and biodistribution of M002 in the HSV-1 sensitive primate *Aotus nancymae* following intracerebral inoculation; and 3) determined whether murine IL-12 produced by M002 was capable of activating primate lymphocytes. Results are summarized as follows: 1) M002 demonstrated superior anti-tumor activity in two different murine brain tumor models as compared to three other genetically engineered HSV-1 mutants; 2) no significant clinical or MRI evidence of toxicity was observed following direct inoculation of M002 into the right frontal lobes of *A. nancymae*; 3) there was no histopathologic evidence of disease in the *A. nancymae* one month or 5.5 years following direct inoculation and 4) murine IL-12 produced by M002 activates *A. nancymae* lymphocytes *in vitro*. We conclude that the safety and preclinical efficacy of M002 warrants the advancement of a $\Delta\gamma_1$34.5 virus expressing IL-12 to Phase I clinical trials for patients with recurrent malignant glioma.

INTRODUCTION

Glioblastoma multiforme (GBM) is both the most common and the most lethal of primary brain tumors. Current treatment consists of surgical resection followed by radiotherapy and chemotherapy (41). However, even with recent advances, the median survival of patients with these tumors is 15 months, with a 2-year survival rate of 26% (50, 51). Thus, there remains a need for new, more potent therapies for GBM and other intracranial malignancies. One such treatment strategy is the use of oncolytic viruses, namely viruses that exhibit tumor-selective replication (39, 48). Among these, herpes simplex virus, type 1 (HSV-1) has been studied as a potential therapy for glioma (4, 28). HSV is well suited for this purpose, as it infects and lyses a variety of cell types and has a well-characterized genome. Moreover, the availability of *in vivo* models allows the study of HSV tumor treatment in a variety of immunocompetent settings. However, because wild type HSV causes potentially life-threatening encephalitis, attenuation is a prerequisite of oncolytic virus development. To this end, mutations within one or more of the following viral genes have been described: thymidine kinase (31), ribonucleotide reductase (U_L39) (22, 34), UTPase (44) or $\gamma_1$34.5 (7, 27). The protein kinase encoded by U_S3 can also be deleted (43) to generate oncolytic HSV (25). In particular, deletion of the diploid $\gamma_1$34.5 gene ablates neurovirulence of HSV (8). The infected cell protein 34.5 (ICP 34.5) is critical for efficient viral replication in normal cells. In infected non-neoplastic cells, protein kinase R (PKR) is stimulated by the production of double-stranded viral RNAs and phosphorylates eukaryotic initiation factor 2 alpha (eIF-2α) to block protein synthesis. Whereas wild-type HSV is able to reactivate eIF-2α and allow viral replication to proceed, $\gamma_1$34.5-deleted HSVs are unable to replicate efficiently in normal cells. In contrast, tumor cells with *ras* overexpression or other deficiencies in the PKR response support the replication of $\gamma_1$34.5-deleted HSV.

The $\gamma_1$34.5-deleted HSV G207, which also contains an inactivating insertion of *lacZ* within the U_L39 gene encoding ICP6 (ribonucleotide reductase heavy chain), has demonstrated efficacy *in vivo* against brain tumors in a number of syngeneic and xenogeneic models of GBM (1, 2, 15, 16, 34), neuroblastoma (53, 54, 56), and meningioma (60). The similar virus HSV1716 is also deleted for $\gamma_1$34.5 but retains the U_L39 gene (26, 32), and has demonstrated efficacy in two different brain

tumor models (19, 24). Both G207 (29) and HSV1716 (36, 45) have been employed in clinical trials and have been demonstrated to be safe following inoculation into tumor tissue as well as the adjacent tissue of the resection cavity (12, 30). The results of these trials demonstrated that while promising responses occurred in some patients, the majority suffered fatal tumor recurrence. Because of tumor heterogeneity and the aggressive growth properties of GBMs, it is likely that tumor eradication will require a multifaceted treatment approach. Also, intratumoral injection of an oncolytic virus is unlikely to result in 100% tumor cell transduction (35), particularly in the case of highly infiltrative neoplasms such as GBM. This fact, and the presence of intratumoral barriers to viral spread, highlights the need for oncolytic HSV that have a greater antitumor effect.

We and others have been investigating the use of oncolytic $\gamma_1$34.5-deleted HSVs as vectors for intratumoral delivery of foreign transgenes (57). Although a variety of transgenes have been shown to increase the efficacy of oncolytic HSVs, our efforts have focused chiefly on cytokine-based gene therapy. We hypothesize that cytokine-expressing oncolytic HSV will, through the induction of an antitumor immune response, mediate enhanced efficacy over non-cytokine HSV and lead to durable antitumor immunity. To this end, we have previously described an oncolytic HSV that expresses murine interleukin 12 (IL-12) under control of the murine early-growth response-1 (egr-1) promoter (37). The pro-inflammatory cytokine IL-12 activates NK cells, mediates T_H1 responses and has additional antiangiogenic properties, and it has been shown to have antitumor effects *in vivo* (9, 10, 52). This IL-12-expressing HSV, designated M002, lacks both copies of $\gamma_1$34.5 but retains U_L39. M002 has been shown to improve survival more effectively than G207 in a murine model of glioma (13). We have also demonstrated that M002 inhibits tumor growth and improves survival more effectively than its non-cytokine parent virus R3659 in syngeneic models of neuroblastoma (38) and glioma (13), and that these effects are mediated by increased infiltration of immune cells.

In the current report, we have determined whether an IL-12-expressing $\Delta\gamma_1$34.5 HSV-1 such as M002 would be suitable for examination in clinical trials for patients with malignant

74 glioma. As such, M002 was assessed for *in vivo* efficacy in experimental murine brain tumor
75 models as compared to other oncolytic HSVs. The safety of M002 following intracranial
76 inoculation was also evaluated in a nonhuman primate model. To summarize our results, we have
77 demonstrated the following: 1) M002 retains sensitivity to acyclovir; 2) M002 replicates more
78 efficiently than G207 in glioma cells; 3) M002 significantly enhances inhibition of tumor growth
79 over time compared to the other HSV-1 mutants tested, in two different models of murine brain
80 tumors; 4) inoculation of purified M002 in the right frontal lobes of *Aotus nancymae* did not
81 produce any significant clinical or MRI evidence of toxicity; and 5) murine IL-12 produced by
82 M002 activates *A. nancymae* lymphocytes in an *in vitro* assay. These data provide additional
83 support for the use of an IL-12 expressing $\Delta\gamma_1$34.5 HSV-1 in a clinical trial for patients with
84 malignant glioma.

85 **METHODS**

86 **Cells**. Vero cells were obtained from the American Type Culture Collection (ATCC,
87 Rockville, MD) and were cultured in Minimal Essential Medium (MEM; Cellgro, Mediatech Inc.,
88 Herndon, VA) containing 7% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) and
89 2 mM L-glutamine (Life Technologies, Carlsbad, CA). The murine 4C8 glioma cell line,
90 syngeneic with $B6D2F_1$ mice, was obtained from C. Dyer (E.K Shriver Center, New York, NY).
91 The human D54MG and U251MG glioma cell lines were provided by D.D. Bigner (Duke
92 University, Durham, NC). The human glioma cell line U87MG was obtained from ATCC, and the
93 glioma xenografts GBM6 and GBM10 (6, 47) were provided by C. D. James (University of
94 California San Francisco, San Francisco, CA). Xenografts were maintained by *in vivo* passaging
95 as previously described, to obtain cells for *in vitro* studies (11). The above lines were maintained
96 in a 50:50 mixture of Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12

(DMEM/F12) supplemented with 2 mM L-glutamine and 7% FBS. All cells were maintained in Corning tissue culture plasticware.

Viruses. HSV-1 "F" strain is a low passage clinical isolate used as the prototype HSV-1 strain for subsequent virus construction (17, 40). Viruses R3659 (23), G207 (34), and R8306, which expresses murine IL-4, (3) are $\gamma_1$34.5-deleted viruses that have been have been described previously. Construction of M002, which expresses murine IL-12 under the transcriptional control of the murine egr-1 promoter, has been detailed elsewhere (38). A Good Manufacturing Practice (GMP)-like lot of M002 was provided by NeuroVir Therapeutics, Inc. (San Diego, CA) and used for neurotoxicity studies.

Acyclovir susceptibility. Vero cells were seeded in 6-well tissue culture plates at 4 x 10^5 cells per well in MEM with 7% FBS and incubated overnight. The cells were then rinsed with phosphate-buffered saline and infected with HSV-1 "F", G207, or M002 at 25 PFU per well in 0.2ml MEM + 1% FBS for two hours at 37 °C, 5% CO^2. Next, 2 ml of acyclovir (Sigma-Aldrich, St. Louis, MO) diluted in MEM + 2% FBS + 0.5% human IgG (Polygam® S/D, Baxter Healthcare Corp., Westlake Village, CA) at 0, 0.03, 0.16, 0.8, 4, 20 and 100 µg/ml was added to each well in triplicate. Plates were incubated for 72 hours at 37 °C, 5% CO_2 and then stained with 0.25% May-Grunwald (Sigma-Aldrich) in methanol (Fisher Scientific, Pittsburgh, PA). The plaques in each well were counted to determine the effective concentration at which 50% (EC50) of the plaques remained versus wells in which no acyclovir was present (42). An EC50 of 10 fold greater than that of wild type virus is considered resistant (46).

Viral Replication. Cells from the human U87MG and U251MG glioma lines and GBM6 and GBM10 xenografts were grown to confluency in 6 well plates and then infected with M002 or G207 at an MOI of 0.1 PFU/cell. The cells were harvested at 24, 48, and 72 h post infection and

then lysed by multiple rounds of freeze/thawing and sonication. Progeny virions in the lysates were titered on monolayers of Vero cells and the average number of PFUs per ml was calculated from quaduplicate wells. Determination of significant differences in titer between M002 and G207 was done by two-tailed, unpaired t-tests.

In vivo tumor responses. All animal experiments were carefully reviewed and approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee and adhered to the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, the National Research Council Guide for the Care and Use of Laboratory Animals and the United States Department of Agriculture (USDA) Animal Welfare Regulations. UAB is licensed as an animal research facility by the USDA and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The animal care and use program at UAB has maintained accreditation with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) since 1971.

Specific pathogen-free female SCID and $B6D2F_1$ mice were obtained from Charles River Laboratories and used at approximately 8 weeks of age. For analysis of efficacy in murine brain tumors, 5×10^5 4C8 or D54MG cells in 5 µl serum-free DMEM/F12 with 5% methylcellulose were stereotactically injected into the right caudal nucleus of B6D2F1 or SCID mice, respectively, using methods detailed previously (7, 37). In the D54MG model, tumors were injected seven days after implantation with 1×10^7 PFU of G207 or M002 in 5 µl sterile saline, or with saline only as a control. Seven days following virus injection, mice that received saline were inoculated with saline. Half the mice in each virus treatment group received an additional 5 µl of the same virus, while the other half received saline only. In the 4C8 model, mice were given intratumoral injections 14 days after tumor implantation of 1×10^7 PFU of R3659, R8306, M002 or saline only. In a second experiment, at seven days after tumor implantation mice were given intratumoral

injections of 1 x 10^7 PFU of G207, M002, or given saline. Seven days after virus injection, mice were re-injected with virus or saline as above. In all experiments, mice were monitored for survival and euthanized when moribund, and necropsies were performed to confirm that tumor growth was the cause of death. Deaths were recorded as described previously (7). Kaplan-Meier survival plots were then constructed and statistical significances in survival between the various cohorts were determined by log-rank (Mantel-Cox) pair wise comparisons.

Toxicity in nonhuman primates. One male and three female adult New World owl monkeys (*Aotus nancymae*) were purchased through the USDA and housed according to the UAB animal husbandry guidelines for nonhuman primates (NHP), thus allowing social interaction between animals. All intracranial inoculations and magnetic resonance imaging procedures were done under conditions of general anesthesia, in which intramuscular ketamine (4 mg/kg) and acepromazine (0.4 mg/kg) were given as sedatives prior to the administration of isoflurane in oxygen (2.4% induction, 1.5-1.8% maintenance). Respiration was assessed by tidal CO_2 monitoring and oxygenation by pulse oximetry. The NHPs were placed in a prone position on a water-controlled heating blanket to maintain body temperature. The head was immobilized in a Kopf stereotaxy, the scalp was clipped and shaved, and the animals were covered with a sterile drape. The skin at surgical site was then sanitized with betadine surgical scrub and an incision was made to expose the skull. After placement of a burr hole 2 mm anterior to the coronal suture and 7 mm lateral to the midline, virus was injected with a Hamilton syringe attached to the stereotaxy into the right frontal lobe of the brain at a depth of 5 mm. Following injection, the burr hole was closed with bone wax and the incision was sutured.

PCR studies of biodistribution following intracerebral inoculation in *A. nancymae*. At both 30 days and 5.5 years post-injection of M002, *A. nancymae* were euthanized by the intravenous administration of 100 mg/kg pentobarbital and tissue samples were collected

aseptically for PCR-based determination of viral biodistribution. Brains were harvested and individual samples were collected from the injection site as well as different sites of both hemispheres (frontal, temporal, and occipital lobes, cerebellum) and the brainstem. Ocular, nasal and oral swabs were collected from the 30 day animal. Additionally, the following tissues were harvested: skin (superficial to injection site), heart, liver, pancreas, stomach, lung, spleen, small intestine, large intestine, adrenal glands (left and right), kidneys (left and right), skin (from site remote to the injection), skeletal muscle, and bladder. DNA was extracted from the tissue samples using a Qiagen EZ1 tissue kit and BioRobot EZ1 Workstation (Qiagen, Valencia, CA) and then amplified by PCR on an ABI 2400 GeneAmp thermal cycler (Applied Biosystems, Carlsbad, CA) using primers specific for HSV gB and polymerase as previously reported (55).

Magnetic resonance imaging. Magnetic resonance imaging (MRI) was performed on anesthetized NHPs at 30 days and 7 months post-inoculation with M002 using a 1.5T Philips (Philips Healthcare, Andover, MA) scanner and knee coil. T1-weighted images were acquired both pre-contrast and post-administration of gadolinium. Also acquired were T2-weighted and fluid attenuated inversion recovery (FLAIR) images.

Histology. All tissue samples in which viral DNA was detected by PCR were analyzed by immunohistochemistry (IHC). This included multiple sites from the brains of NHPs euthanized 30 days and 5.5 years post-inoculation of M002, as well as a sample of spleen from the 30 day animal. Formalin-fixed paraffin embedded sections 10 µm thick were stained with hematoxylin and eosin. Additional sections 6 µm thick were prepared for IHC staining by deparaffinization in xylene, rehydration through graded alcohols, and antigen retrieval with pH 6.0 sodium citrate buffer. Endogenous peroxidases were quenched with hydrogen peroxide and blocking was done with horse or goat serum (Vector Laboratories, Inc., Burlingame, CA), depending upon the antibody to be used. HSV antigen staining was done using a rabbit polyclonal antibody (BioGenex

Laboratories, Inc., Fremont, CA) diluted 1:400. T cells were detected with a mouse anti-CD3 monoclonal antibody (Ventana Medical Systems, Tucson, AZ) which was supplied ready to use. Staining for macrophages was done using a mouse monoclonal antibody diluted 1:100 (MAC387; AbD Serotec, Raleigh, NC) or as provided by the manufacturer (HAM-56; Cell Marque Co., Rocklin, CA). Secondary antibodies specific for rabbit or mouse IgG were supplied as Vectastain ABC kits (Vector Laboratories). Slides were developed using a Vector VIP (Vector Laboratories) peroxidase substrate detection kit and counterstained with methyl green. All slides were examined by a board-certified neuropathologist.

Primate Lymphocyte Activation. The responsiveness of *A. nancymae* lymphocytes to the murine IL-12 expressed by M002 was assessed by a lymphocyte re-activation assay. Briefly, peripheral blood lymphocytes were isolated from pooled blood samples harvested from anesthetized *A. nancymae.* The cells were cultured for 72 h in RPMI-1640 with 10% (v/v) FBS, supplemented with 2.5 µg/ml phytohemagglutinin (PHA), 1000 U/ml recombinant interferon gamma and 50 µM β-mercaptoethanol. The cells were then plated in 96-well tissue culture plates at 2.25×10^4 cells/well and incubated in the absence of PHA for 24 h. Next, the cells were cultured for 72 h in RPMI-1640 medium with 10% (v/v) FBS and 50 µM β-mercaptoethanol and replicate wells were stimulated with a range of dilutions of either PHA (0.01 – 32 µg/ml) as a control, or with dilutions of recombinant murine IL-12 (1.25 – 40 ng/ml) obtained from the concentrated conditioned medium of M002-infected Vero cells. The cells were then pulsed with tritiated thymidine (^{3}[H]-Tdr) at 1 µCi/well for 21 h. Lymphocyte activation, as determined by incorporation of ^{3}[H]-Tdr into the cellular DNA, was assessed by scintillation count and expressed as counts per minute (cpm).

RESULTS

M002 retains sensitivity to acyclovir. To verify the sensitivity of M002 to acyclovir, a plaque reduction assay was performed. Sensitivity was compared to wild-type strain "F", the parent virus of M002, and G207, a virus previously used in North American clinical trials (29, 30). M002 exhibited sensitivity to acyclovir at levels similar to wild-type virus (**Figure 1**). These data translate into an EC50 of <1.0 μg/ml for M002, a value similar to wild type isolates tested in our laboratory (58).

M002 replicates more efficiently than G207 in human glioma cells. Next, we compared the abilities of M002 and G207 to replicate in glioma cells. Monolayers of the human glioma lines U87MG and U251MG or cells derived from the xenografts GBM6 and GBM10 were infected with M002 or G207 at an MOI of 0.1 PFU/cell. At multiple time points post infection (24, 48, and 72 h), viral replication was determined. As shown in **Figure 2**, M002 replicated to a titer 1 – 2 log higher than that of G207 in both the established cell lines and the xenograft-derived cells. This enhanced replication was seen across all three time points.

M002 improves survival more effectively than other cytokine-expressing HSV in a murine brain tumor model. The anti-tumor efficacy of M002 was next compared directly with the anti-tumor efficacy of R8306, a $\gamma_1$34.5-deleted HSV-1 that expresses IL-4. R8306 was previously shown to be an effective antiglioma agent in a preclinical murine brain tumor model (3). To compare these viruses, the murine 4C8 tumor model was employed. These cells form invasive brain tumors *in vivo*, with histological features typical of human gliomas (13). M002 replicates to similar levels in 4C8 as in human glioma cells, and expresses physiological amounts of IL-12 (13). Intracranial tumors were established in B6D2F$_1$ mice and then treated after 14 days with M002 or R8306. Control cohorts of mice were given saline or treated with the non-cytokine-expressing virus R3659, an oncolytic HSV with a diploid $\gamma_1$34.5 deletion but lacking the U_L39

deletion mutation present in G207. The mice were monitored for survival for 84 days, at which point surviving mice were euthanized. Treatment with M002 resulted in a significantly increased median survival time (MST) (p <0.05) when compared to either R8306 or the control virus R3659 (**Figure 3)**. Whereas mock-treated mice exhibited an MST of 52 days, mice treated with either R3659 or M002 had MSTs of 69 days and > 84 days, respectively. Although mice treated with R8306 had a shorter MST (31 days) than mock-treated, the R8306 group had the second greatest proportion (40%) of long-term survivors after the M002 group (80%). In a similar experiment conducted in A/J mice bearing syngeneic Neuro-2A murine neuroblastoma tumors, M002 also improved survival more effectively than M004, a similar HSV that expresses granulocyte-macrophage colony-stimulating factor (unpublished data). Together, these results provide evidence supporting the use of IL-12 as a rational choice among other cytokine-expressing HSVs for clinical use.

M002 is superior to G207 in xenogeneic and syngeneic models of glioma. In a syngeneic murine model of glioma, M002 improves survival more effectively than G207 following a single intratumoral injection of virus (13). M002 is also more effective than R3659 in both syngeneic (13) and xenogeneic (49) models of glioma. We next sought to directly compare the anti-tumor activity of M002 with G207 in human glioma xenografts *in vivo*. Multiple dosing was also assessed for potential ability to further enhance efficacy.

To compare the effects of treatment with M002 and G207 against human glioma cells *in vivo*, a survival study was conducted in SCID mice implanted orthotopically with human D54MG xenografts. These cells form relatively well-circumscribed brain tumors in vivo that grow rapidly by expansion. Viral cytotoxicity and IL-12 expression by M002 has previously been confirmed in this line (37). Mice were treated after seven days with G207 or M002, or given saline only as a

control. Seven days after the first injection, half of the mice in each treatment group received a second injection of the same virus, while the other half was given saline only. A single injection of G207 did not significantly improve survival versus mock-treated, with MSTs of 22 days and 18 days, respectively ($p = 0.157$) (**Figure 4**). A second dose of G207 did not further improve survival (MST of 22 days; $p = 0.0754$ vs. mock, 0.429 vs. single dose). In contrast, treatment with M002 significantly improved survival over that of mock treatment. A single dose resulted in an MST of 29 days ($p = 0.00254$), and two doses resulted in an MST of 30 days ($p = 0.000473$). The differences in survival between two doses ($p = 0.0002$) of G207 and M002 was also significant.

In similar multiple dosing study of M002 versus G207 conducted in an immunocompetent intracranial model of glioma, G207 was less effective than M002 against 4C8 tumors (data not shown). Whereas the MST of mock-treated mice was 39.5 days, those treated with G207 exhibited MSTs of 60 days (single dose) and 49 days (two doses). M002-treated mice survived longer when either a singe dose (MST = 71.5 days) or two doses (MST = 94 days) were given. Overall, these results indicate that M002 is more effective against human glioma xenografts than G207, and that additional doses may enhance the antitumor effect.

M002 does not produce encephalitis or other significant effects in primate toxicity studies. Intracerebral inoculation of G207 in *Aotus nancymae* primates, a New World monkey highly susceptible to HSV-1 replication (18, 33), does not result in virus-associated toxicity (14, 55). However, whether this same safety profile would be exhibited by an IL-12-expressing HSV such as M002 was unclear. Thus, to verify safety in a nonhuman primate model, M002 was directly inoculated into *A. nancymae* brains. A preparation of M002 that was made according to clinical Good Manufacturing Practice specifications was inoculated into the monkeys at doses up to 4.8×10^8 PFU. No clinical evidence of toxicity was observed in any of the animals, as assessed

by changes in temperature, neurologic performance, feeding or social behavior, or weight. Examination of injected animals by MRI both one month and seven months post-injection demonstrated a lack of encephalitis or any other toxicity (**Figure 5**). One female animal, which had been injected in the right frontal lobe with 4.8 x 10^8 PFU of M002, was euthanized one month post-injection for histological evaluation (**Figure 6**). Overall, the areas of the brain examined (left and right frontal, temporal and occipital lobes; brainstem, cerebellum) appeared normal, with a few potentially study-related features noted as follows. The left temporal lobe showed a single perivascular chronic inflammatory cell cuff, deep at the grey-white junction. In the superficial left frontal lobe, a focal encephalitis with active astrogliosis, glial nodules and neuronphagia was observed. Accordingly, the presence of CD3+ T cells was confirmed by immunostaining. Rare perivascular lymphocytes were observed along the wound tract. No macrophage or HSV antigens were detected by immunostaining in any part of the brain. Five and one-half years after inoculation, one of the injected females, along with a cagemate that had not been treated with virus, was found to demonstrate delayed type hypersensitivity to purified protein derivative on routine tuberculosis surveillance monitoring. Both animals were euthanized and necropsied. No evidence of mycobacterial infection was found, and no evidence of toxicity related to M002 treatment was seen in the treated animal. Overall, the sections of the brain examined, including the injection site, appeared normal. Reactive changes, which may have been age-related or treatment-related, were observed in some areas of the brain. These changes were deemed clinically insignificant. No HSV or CD3+ cells were detected in any part of the brain. An additional treated female is now more than ten years status post injection and remains alive and healthy. One male *A. nancymae* was injected with a lower dose of M002 (1 x 10^8 PFU non-purified standard laboratory preparation). This animal died under anesthesia during follow-up MRI three days after injection of virus. No abnormalities were observed by MRI. A post-mortem

necropsy revealed that subclinical naturally-occurring glomerulonephritis and moderate bronchopneumonia contributed to anesthetic complications. The brain was fixed and serially sectioned, then stained both by hematoxylin and eosin as well as by IHC for HSV antigens. Regardless of histopathologic approach, no evidence of HSV encephalitis or virus-related toxicity was found. No hydrocephalus or ventriculomegaly was present in any animal.

M002 DNA can be demonstrated in *A. nancymae* after inoculation in both short and long-term treated animals. To examine the persistence and biodistribution of M002 following intracerebral inoculation, it was necessary to confirm that copies of HSV DNA were present in *A. nancymae* brain after treatment and to assay for HSV DNA in other organs. Multiple sites within the brain, multiple organ sites, and (in the 30 day animal), swabs to evaluate potential shedding sites, were all tested for HSV DNA by PCR. Notably, HSV DNA was present in both the 30 day and the 5.5 year animal at multiple sites of the forebrain and brain stem, but not the cerebellum (**Table 1**). In the 30 day animal, HSV DNA was present in the skin at the inoculation site and in the nasal swab, but not in the ocular or oral swabs. Systemically, HSV DNA was detected in the spleen but could not be detected in any other major organ. In the 5.5 year animal, no samples outside the brain tested positive for HSV DNA. HSV antigens were not detected by IHC in any sample at either time point. These findings are consistent with previous studies with G207 in patients, in which HSV DNA can be detected by PCR up to 157 days post-inoculation, but HSV is not detectable by IHC (29).

Murine IL-12 is biologically active in *Aotus nancymae.* To confirm that *A. nancymae* lymphocytes respond to murine IL-12 produced by M002, a lymphocyte activation assay was performed using pooled lymphocytes. Lymphocytes were activated in the presence of PHA, murine recombinant IL-12 or medium alone. As determined by ^{3}[H]-Tdr incorporation, the lymphocytes showed similar activation to murine recombinant IL-12 as to PHA (**Figure 7**). These

results confirm that the lack of toxicity seen in M002-injected *A. nancymae* is not due to a failure of the primate lymphocytes to recognize murine IL-12.

DISCUSSION

Oncolytic HSVs are being actively investigated as potential anti-glioma therapies. HSVs can be neuroattenuated by a number of strategies, with deletion of the diploid $\gamma_1$34.5 gene being the approach taken for two different HSVs employed in clinical trials. Overall, the results of these trials have underscored the need for HSVs with improved antitumor effects. Our research group has investigated the potential use of oncolytic HSVs as a platform for the delivery of foreign transgenes. Our efforts have focused mainly on cytokine delivery, under the general hypothesis that a cytokine-expressing HSV will activate an antitumor immune response that will lead to the elimination of non-infected tumor cells. The properties of IL-12 warranted its consideration for introduction into an oncolytic HSV and subsequent evaluation as an anti-tumor agent. IL-12 activates both cytotoxic T lymphocyte and T_H1 responses by stimulating interferon gamma production from both NK cells and T cells. Furthermore, by NK cell activation and subsequent cytokine secretion, IL-12 also stimulates an anti-angiogenic effect against tumor vasculature. M002 was engineered to express murine IL-12 (38). While murine IL-12 exhibits biological activity on human lymphocytes, human IL-12 does not activate murine lymphocytes. Thus, any construct designed to examine the effects of IL-12 in a murine system must necessarily utilize murine IL-12. As a consequence of its multiple antitumor activities, M002 has been shown to mediate a superior antitumor effect versus other non-cytokine HSVs (13, 37, 49).

In the current study, we sought to pre-clinically evaluate the suitability of M002 for use against glioma in clinical trials. First, it was important to demonstrate that M002 was susceptible to an antiviral drug routinely used in the therapy of HSV infections. In a plaque-reduction assay,

M002 replication was inhibited by acyclovir to a similar extent as the parent HSV-1 strain "F". These data indicate that neither the expression of IL-12 nor other possible uncharacterized genetic differences from F strain have caused M002 to lose sensitivity to acyclovir. Although the establishment of encephalitis by a $\gamma_1$34.5-deleted HSV has never been documented in humans, the ability to control M002 replication pharmacologically serves as an important backup safety mechanism.

Next, the antitumor activity of M002 was directly compared *in vivo* in immunocompetent mice to another cytokine-expressing HSV, R8306. In previously published studies, we have shown that M002 stimulates the intratumoral infiltration of immune cells, including CD4, CD8, NK and macrophages (13, 37) in both the N2A (37) and 4C8 models (13). Since we have also previously shown that R8306, which expresses IL-4, is effective against glioma in an immunocompetent model (3), we next wanted to compare the efficacy of that virus to M002. Here, we have shown that M002 treatment was superior to that of R8306, leading to a higher proportion of long-term survivors. We speculate that this difference may be due to the differential effects of the respective cytokines. Both R8306 (3) and M002 (38) stimulate an influx of CD4+ and CD8+ T cells and macrophages, but M002 also stimulates NK cell infiltration (13). Furthermore, IL-4 stimulates the differentiation of T_H2 cells, whereas IL-12 stimulates a T_H1 response. Finally, the anti-angiogenic effects of IL-12 may have also contributed to the increased efficacy of M002 versus R8306, as it has been reported that IL-12 expression from an oncolytic HSV reduces tumor angiogenesis (59). While M002 treatment resulted in 80% long term survival in this model, some of the mice eventually succumbed to tumor growth. This may have been a consequence of incomplete viral spread throughout these infiltrative tumors.

In additional experiments, M002 treatment was also superior to that of G207 in both immunodeficient and immunocompetent models. In the first, M002 was more effective than G207

against human xenografts in SCID mice. Our *in vitro* replication data indicate that M002 replicates to higher titer in glioma cells than does G207. The superior antitumor effect of M002 over G207 in the immunodeficient model is likely due to its enhanced replicative ability, since adaptive immune responses are not elicited and thus not able to contribute to the antitumor effect. Unlike the immunocompetent model, in which long-term survivors were observed, in the immunodeficient model all animals eventually died of tumor burden, probably a result of incomplete local virus replication that allowed surviving tumor cells to continue growth. Other factors that may have contributed to the enhanced efficacy of M002 relative to G207 in this model include the anti-angiogenic properties of IL-12 and NK cell stimulatory effects. Also, the U_L39 gene retained by M002 may confer a replication advantage over G207, which lacks this gene, in this particular cell line. In this model, an additional dose of M002 did not lead to a statistically significant increase in efficacy over a single dose. This may be evidence that in the absence of a potential antitumor effect mediated by the adaptive immune system, innate antiviral responses may offset the potential benefit of additional virus. In an unpublished experiment conducted in immunocompetent mice, we observed that a single injection of M002 was superior to a single injection of G207, and that an additional dose of virus further extended the benefit of M002 treatment but did not extend the benefit of G207 treatment. In this case, it is possible that an anti-viral immune response prevented an increase in efficacy for a double dose of G207, but the many antitumor effects of IL-12 expression may have allowed for double dosing of M002 to overcome potential antiviral responses to result in an overall increase in efficacy. The potential benefits of multiple dosing with M002 may simply be model dependent and/or require a greater number of mice (more power) to demonstrate, and further clarification of this issue is needed. However, previous murine studies have shown that the increase in immune cell infiltration mediated by M002 may begin to wane seven days after treatment. In the clinical setting, in which treatment

schedules occur over a longer time frame (i.e., months), multiple dosing may still increase efficacy.

All of the efficacy experiments in this report were conducted with the same viral dose (1 x 10^7 PFU), which was based upon previous work in which we determined maximum tolerated dose (PFU/LD_{50}) of M002 in the HSV-sensitive A/J mouse strain to be 2 x 10^7 PFU (Parker *et al*., 2000). We therefore routinely use 1 x 10^7 PFU as the highest dose which can be used without risk of toxicity. Whether smaller doses of virus would also be effective remains to be determined, although given the replicating nature of these constructs, the concept of a minimally effective dose is subject to debate. Regarding the dose to be used in a clinical trial, rather than try to determine starting doses based upon murine data, we will take advantage of prior human data with G207 and HSV 1716. HSV 1716 showed no toxic responses at up to 1 x 10^5 PFU (45), whereas G207 was used at even higher doses (3 x 10^9 PFU) (29). Neither study established a maximum tolerated dose. We propose that doses in a clinical trial with an IL-12-expressing HSV begin at 1 x 10^5 PFU and increase to 1 x 10^9 PFU in the absence of limiting toxicities. The highest likely clinical dose is therefore 100 times higher than the dose used in the murine studies described here. However, given that the intracranial volume of the human is approximately 2000 fold greater than that of a mouse, the relative dose to be used in a clinical trial is approximately 20 fold less than what has been used in the mouse experiments.

To elucidate the potential safety profile of M002 in a readily translatable model, we evaluated the toxicity and biodistribution of M002 following intracerebral viral injection in the nonhuman primate *Aotus nancymae*. Similar studies have previously been conducted with G207 (14, 55). Over the course of this study, no toxicities related to M002 injection were observed, either clinically or histologically. While mild inflammation was present at the site of inoculation in the 30 day post-treatment *A. nancymae* specimen, other major pathologic features were not

observed, and no pathological changes were detected by MRI. These observations are similar to those following intracerebral injection of G207, in which mild inflammatory changes occurred at the injection site, with no MRI-based evidence of toxicity (14), although the dose of G207 used (10^9 PFU) was higher than the highest dose in this study (4.8×10^8 PFU). We observed infiltration of CD3+ T cells but did not observe HSV-positive cells as reported by Hunter *et al*. (14), although our examination was conducted at 30 days rather than 5 days post injection. We did not directly assay for IL-12 (either murine or endogenous) in this experiment, but given the lack of HSV staining and the relatively mild immune response, we speculate that presence of IL-12 would have been difficult to detect. Also, no signs of IL-12 toxicity were observed in any animal. In this study, the long-term safety of M002 treatment is suggested by the lack of toxicities seen in the 5.5 year animal, and further supported by the continued health of the surviving M002-treated animal, now at 10 years post-injection. Previously, the safety of G207 administration in *A. nancymae* had been followed out to 41 months (14). Regarding viral persistence and biodistribution, both short term and long term animals in this study showed retention of HSV DNA in the brain and at the inoculation sites, consistent with the ability of $\gamma_1$34.5-deleted viruses to undergo latency but not emerge from latency (20). This is not altogether surprising, as tumor resected from malignant glioma patients greater than 150 days post-treatment with G207 has been positive for HSV DNA (29). It has also been reported that HSV DNA can be detected in autopsy specimens from the brains of normal humans who were seropositive for HSV (5). We observed HSV DNA at multiple sites throughout the brain but not the cerebellum, a finding also noted in the G207 study by Todo *et al*. (55), who reported the presence of HSV DNA 2 years post injection. Although G207 was not detected outside of the brain (55), HSV DNA was present in the spleen of the 30 day animal in this study. We also detected HSV DNA in a nasal swab at 30 days and in the skin superficial to the injection site, neither of which were assayed in the G207 study. Both of these sites were

453 negative for HSV at 5.5 years, however. Since HSV antigens were not detected by IHC in any of
454 these samples, the presence of DNA may be a product of immune cell clearance or a very low
455 level of retained virus. The significance of this finding remains to be determined. However, it
456 should be noted that in the G207 Phase I study mentioned above, viral antigens similarly were not
457 detected by IHC at five months post-injection, in spite of PCR-based detection of viral DNA (29).
458 In total, the distribution of M002 after intracerebral injection is not markedly different from that of
459 G207.

460 If murine IL-12 was not functional in *A. nancymae,* the results of the toxicity study would
461 be less meaningful. Presumably, with function being retained across species from mouse to
462 human, murine IL-12 would be expected to function in the *A. nancymae* as well as in mice. A
463 study conducted by Kim *et al*., demonstrated that murine IL-12 expressed from an injected DNA
464 plasmid was biologically active in rhesus macaques, another nonhuman primate species similar to
465 *A. nancymae* (21). We have confirmed here that murine IL-12 activates *A. nancymae*
466 lymphocytes, validating the safety profile of M002 in this model.

467 In conclusion, we have conducted a pre-clinical determination of whether an IL-12-
468 expressing $\gamma_1$34.5-deleted HSV-1 might be a suitable virus to advance into clinical studies for
469 patients with malignant brain tumors. To summarize our results: 1) M002 mediates efficacy in
470 preclinical brain tumor models superior to that of other mutant HSV-1, including one currently
471 under clinical evaluation; 2) No significant toxicity is seen after intracerebral inoculation of M002
472 into mice or the HSV-sensitive primate *A. nancymae* despite long-term persistence of viral DNA
473 as measured by PCR; and the potential for acyclovir treatment and patient rescue exists should
474 such toxicity occur; 3) Murine IL-12 expressed by M002 activates *A. nancymae* lymphocytes, thus
475 confirming the validity of this model for evaluation of safety. Findings consistent with efficacy
476 greater than G207, lack of significant neurologic and systemic toxicity by clinical, radiologic, and

pathologic evaluation despite long-term persistence of HSV DNA, and confirmation of the activity of IL-12 produced by the virus on *A. nancymae* lymphocytes all support the advancement of an IL-12-expressing $\gamma_1$34.5-deleted HSV-1 into clinical trials for patients suffering from malignant glioma. Although species specificity of human IL-12 prevents its use in murine model, we have also generated a syngeneic virus that expresses human IL-12. This virus might be appropriate for use in clinical trials, to avoid the possibility of any unforeseen differences in activity between human and murine IL-12, and possible generation of an immune response against a murine protein when used in human patients.

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FIGURE LEGENDS

Figure 1. Replication of the IL-12-expressing γ34.5-deleted HSV M002 is inhibited by acyclovir. In a plaque-reduction assay, M002 was found to have an EC50 of < 1.0 μg/ml, similar to its wild-type parent "F" as well as G207, another γ34.5-deleted HSV-1 mutant already used in clinical trials. Shown are the proportions of surviving plaques and standard deviations at varying concentrations of acyclovir, relative to infected control cells grown without the drug.

Figure 2. M002 replicates more efficiently than G207 in glioma cells. Monolayers of the human glioma cell lines U87MG (**A**) and U251MG (**B**), and cells derived from glioma xenografts GBM6 (**C**) and GBM10 (**D**), were infected with M002 (filled boxes) or G207 (open boxes) at 0.1 PFU per cell, and viral replication was assayed at the indicated times. No G207 progeny were recovered from GBM10 at 72 h. Shown are the averages and standard deviations of quadruplicate wells at each time point. Significant differences in titer at each time point are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$.

Figure 3. Survival of $B6D2F_1$ mice following intracranial injection of syngeneic 4C8 murine glioma cells. 14 days after tumor implantation, the mice were inoculated with saline (open triangles) or 1 x 10^7 PFU of either IL-12-expressing M002 (filled circles), IL-4-expressing R8306 (open circles), or the control virus R3659 (filled triangles). Median survival was highest in the M002 treated group (>84 days, $p < 0.05$), followed by the R3659 group (69 days), saline group (52 days), and the R8306 group (31 days). The R8306 group had the second greatest proportion of long-term survivors, after M002.

733 **Figure 4.** Superior efficacy of M002 versus G207 against intracranial human glioma xenografts.
734 SCID mice implanted with D54-MG cells were injected 7 days after tumor establishment with
735 1 x 10^7 PFU of M002 or G207, or with saline only. Seven days later, mice that received saline
736 were re-injected with saline. Half of the mice that received virus received an additional 5 µl of the
737 same virus, and the other half received 5 µl of saline. Significant differences in median survival
738 times (MST) were as follows: saline/saline vs. M002/saline (p = 0.00254), vs. M002/M002 (p =
739 0.000473); M002/saline vs. G207/G207 (p = 0.00149); M002/M002 vs. G207/G207 (p = 0.0002).
740
741 **Figure 5.** Shown are MRI images of two different *A. nancymae* after inoculation with M002, *a)*
742 *Animal 1***.** One month prior, 1.2 x 10^8 PFU of M002 was inoculated in the right frontal lobe.
743 Images are in axial plane. Shown are i) FLAIR, ii) T2-weighted, iii) T1-weighted pre-gadolinium,
744 and iv) T1 post-gadolinium images. *b) Animal 2.* 7 months prior, 4.8 x 10^8 PFU of M002 was
745 inoculated in the right frontal lobe. Images are in coronal plane and inverted. Shown are i) FLAIR,
746 ii) T2-weighted, iii) T1-weighted post-gadolinium, and iv) additional plane, T1 post-gadolinium
747 images. No pathologic changes are seen after M002 administration.
748
749 **Figure 6**. Shown are micrographs of *A. nancymae* brain 30 days after injection with M002, stained
750 with hematoxylin and eosin **(A and B)** at low **(4X, A)** and higher (**20X, B)** magnification. Note
751 that while mild inflammatory changes are present, as would be expected from local expression of
752 IL-12, no significant neuronal loss or glial scarring is seen. Also shown is a section stained by
753 immunohistochemistry for CD3-positive cells at two different magnifications **(20X, C and 40X,**
754 **D)**.
755

Figure 7. The ability of *A. nancymae* peripheral blood lymphocytes to respond to recombinant murine IL-12 was assessed based on incorporation of 3[H]-Thymidine into cellular DNA. Pooled lymphocytes were stimulated with dilutions of concentrated supernates from Vero cells infected with M002 (IL-12), or with PHA as a control, and then pulsed with ^{3}[H]-Tdr. Activation was assessed by scintillation count and is expressed as counts per minute (CPM), plotted in comparison to a linear IL-12 response.

764

Table 1: Results of PCR Testing for HSV DNA in treated *nancymae* at 30 days and 5.5 years post-injection.

Location	30 Days	5.5 Years
Frontal Lobe L	+	+
Frontal Lobe R	+	+
Temporal Lobe-L	+	+
Temporal Lobe-R	+	+
Parietal L	ND	+
Parietal R	ND	+
Occipital L	+	-
Occipital R	+	+
Cerebellum L	-	-
Cerebellum R	-	-
Pons	ND	+
Brain Stem	+	+
Wound Inj. Site	+	+
Ocular Swab	-	ND
Nasal Swab	+	ND
Oral Swab	-	ND
Heart	-	ND
Liver	-	ND
Pancreas	-	-
Stomach	-	ND
Lung	-	ND
Spleen	+	-
S. Intestine	-	-
L Intestine	-	-
L. Adrenal	-	-
R. Adrenal	-	-
L. Kidney	-	-
R. Kidney	-	-
Skin	+	-
Skeletal Muscle	-	-
Bladder	-	-

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