

Targeted Therapy for Glioblastoma Multiforme Neoplastic Meningitis with Intrathecal Delivery of an Oncolytic Recombinant Poliovirus

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Abstract Purpose: The toxicity and antitumor activity of regional intrathecal delivery of an oncolytic recombinant poliovirus, PVS-RIPO, was evaluated in rodent models of glioblastoma multiforme neoplastic meningitis.

Experimental Design: To evaluate for toxicity, PVS-RIPO was administered into the spinal cord of transgenic mice that express the human poliovirus receptor, CD155, and into the intrathecal space of athymic rats without tumor. To evaluate efficacy, two different doses of PVS-RIPO were administered intrathecally 3 days after athymic rats were inoculated intrathecally with an aggressive human glioblastoma multiforme xenograft.

Results: No clinical or histologic evidence of toxicity was found. In efficacy studies, median survival was increased by 174.47% from 8.5 days in the group treated with UV light-inactivated virus to 15 days in the rats treated with 1.0×10^7 plaque-forming units (pfu) of PVS-RIPO ($P < 0.0001$). A similar increase in median survival was seen in the group receiving 1.0×10^9 pfu PVS-RIPO ($P < 0.0001$); however, there was no statistically significant dose-response relationship ($P = 0.345$). In addition, 1 of 10 rats in lower-dose PVS-RIPO – treated group and 3 of 10 rats in higher-dose PVS-RIPO – treated group survived >60 days after tumor cell inoculation and had no evidence of residual tumor at autopsy.

Conclusion: These results suggest that intrathecal treatment with PVS-RIPO may be useful for treatment of neoplastic meningitis in patients with glioblastoma multiforme and provides a rationale for clinical trials in this area.

Despite aggressive surgical resection, radiotherapy, and chemotherapy, patients with glioblastoma multiforme suffer persistent recurrences that frequently culminate in leptomeningeal spread (1). Because systemically delivered therapeutics penetrate the intrathecal compartment poorly and because only a limited number of agents are approved for intrathecal use, treatment strategies for neoplastic meningitis have been limited (2–9), and novel strategies are desperately needed (10–12). Oncolytic viruses that are being developed for intraparenchymal solid tumors, however, may provide a unique tool for therapy of neoplastic meningitis as well (13). Although a great number of viral agents are under consideration for use in such strategies (14–23), an attenuated poliovirus has a number of intrinsic properties that render it particularly suitable for

targeting neoplastic lesions within the central nervous system (CNS; ref. 24), including a natural propensity to invade the subarachnoid space and destroy CD155-expressing cells in this compartment (25).

Poliovirus is a nonenveloped, plus-stranded RNA virus belonging to the Picornaviridae family and is the causative agent of paralytic poliomyelitis. In poliomyelitis selective targeting of motor neurons is determined by the distribution of the poliovirus cellular receptor (26), the immunoglobulin superfamily molecule, CD155 (27). We have previously shown that a number of tumors, including glioblastoma multiforme, also express this cellular receptor making it an ideal tumor associated target for oncolytic viral therapy (13, 28). Moreover, CD155 recently has been identified as a key determinant of tumor cell migration, invasion, and metastasis (29), and transcriptional activation of the *CD155* gene has been linked to signaling pathways commonly deregulated in malignancy, including *sonic hedgehog/gli* (30), fibroblastic growth factor, and oncogenic *ras* (31).

To apply poliovirus to tumor therapy, we eliminated its inherent neuropathogenicity by manipulating an essential *cis*-acting regulatory element involved in gene expression control (32). Poliovirus, like all picornaviruses, uses an internal ribosomal entry site contained within its extensively structured 5' untranslated region to initiate translation in a cap-, 5' end-independent manner (33, 34). Replacing the cognate poliovirus internal ribosomal entry site with its counterpart from human rhinovirus type 2 eliminated the virus' ability to cause

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poliomyelitis in mice transgenic for CD155 and *Cynomolgus* macaques (32, 35). However, this alteration does not reduce the cytopathogenicity of the lytic virus for malignant cell types that express CD155, such as glioblastoma multiforme.

In this study, we evaluated the toxicity and efficacy of intrathecal administration of a novel clinical grade recombinant poliovirus, PVS-RIPO, developed through the NIH Rapid Access to Inventional Development program, in transgenic mice expressing the human poliovirus receptor, CD155, and in an athymic rat model of human glioblastoma multiforme neoplastic meningitis. Intraspinal and intrathecal administrations of PVS-RIPO were found to be nontoxic in both the transgenic mice and athymic rats. PVS-RIPO was also highly effective against human glioblastoma multiforme xenografts growing in the subarachnoid space of athymic rats.

Materials and Methods

Cell line. The human glioblastoma multiforme cell line, U87MGΔEGFR, was obtained from W. Cavenee (University of California at San Diego; ref. 36). These cells are grown in zinc option medium with 0.23% HEPES, 0.22% NaCO₃, and 10% fetal bovine serum.

Preparation and inactivation of virus. PVS-RIPO was derived from a previous version of the recombinant virus, PV1(RIPO), by substituting the coding region of poliovirus type 1 (Mahoney) with its counterpart from the type 1 live attenuated Sabin [PV1(S)] vaccine strain. The synthesis of PVS-RIPO was reported previously (13). In brief, infectious PV1(S) cDNA [clone pS1 (T7)], kindly provided by A. Nomoto (University of Tokyo, Japan), was digested with *Ava*I. The resulting 7.0-kb restriction fragment was ligated with a PCR fragment amplified from PV1 (RIP0S) using primers (a) 5'-GGGTCCGACTAATACGACTCC-TATAGTAAACAGCTCTGGGGTTGT-3' and (b) 5'-CCATTTCTCGGG-CACTGGAGCG-3' and pBS vector DNA (New England Biolabs, Beverly, MA) digested with *Ava*I and *Sal*I. PVS-RIPO cDNA was processed for rederivation of virus as described before (32).

Virus inactivation by UV irradiation was carried out in a Stratagene UV-linker according to previously established procedures. The absence of virus infectivity after UV treatment of samples was confirmed by plaque assay as described below.

Plaque assay. Cell monolayers, grown in DMEM with 10% fetal bovine serum, penicillin (10 units/mL), and streptomycin (100 µg/mL), were inoculated with a viral suspension at a multiplicity of infection of 10 and incubated for 30 minutes at room temperature. Culture dishes were thoroughly rinsed with five changes of growth medium to remove unbound particles and plated at 37°C thereafter. At the indicated intervals, culture dishes were removed and treated with three consecutive freeze/thaw cycles. Virus titers in infected monolayer cultures were quantified by plaque assay as described previously (24). The PVS-RIPO titers that we used in this study were determined based on our previous preclinical studies in experimental animals evaluating i.t. delivery of the agent (13, 24).

Antigen capture analysis of CD155 expression. The method of antigen capture for CD155 has been reported previously (13, 28). In brief, cells were grown in monolayer culture and homogenized in lysis buffer. The protein concentrations in cell lysates were determined using the Bradford method. ELISA plates were treated overnight with monoclonal anti-CD155 antibody, D171, and suspended in carbonate buffer. Treated plates were rinsed thrice with PBS and individual wells were filled with cell lysate diluted with PBS to achieve the desired protein concentration. Plates were incubated for 4 hours at room temperature while gently rocking. Subsequently, the wells were thoroughly rinsed thrice with PBS and treated with SDS-PAGE loading buffer at 95°C to release the captured antigen. The recovered material was analyzed by SDS-PAGE, transferred to nitrocellulose membranes,

and subjected to Western blot. Filters were blocked overnight in TBST [10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20] containing 3% dry milk. After treatment with the polyclonal anti-CD155 antibody, D480 (diluted in TBST, 3% dry milk at 1:1,000) for 1 hour at room temperature, the blots were rinsed thrice in TBST and incubated for 1 hour at room temperature with secondary biotinylated anti-rabbit IgG antibody (diluted in TBST, 3% dry milk at 1:500; Vector Labs, Burlingame, CA). After three washes with TBST, the filters were treated with streptavidin-horseradish peroxidase complex (diluted in TBST, 3% dry milk at 1:2,000; Roche, Indianapolis, IN) and developed with a chemoluminescent substrate (enhanced chemiluminescence; Amersham, Arlington Heights, IL).

Toxicity study in CD155 transgenic mice. The evaluation of neurovirulence of poliovirus strains in CD155 transgenic mice has been described previously (37). Serotype 1 PV reference strain (WHO/1) and two clinical lots of PVS-RIPO (0402018, 2nd passage; 0404007, 5th passage in Vero cells) were administered intraspinally to CD155 transgenic mice (TgPVR21) and neurologic symptoms were evaluated using a standard clinical score as follows: grade 0, no neurologic symptoms; grade 1, paralysis of limb; grade 2, death. Evaluation of neurologic symptoms was done from days 0 to 14 after intraspinal PVS-RIPO inoculation. The rodent model for paralytic poliomyelitis in TgPVR21 mice was developed as a replacement for the traditional monkey neurovirulence assay in *Cynomolgus* macaques. It is a rigorous and standardized clinical assay. It has been established that clinical observation alone is sufficient to predict the neurovirulent potential of live attenuated poliovirus strains, as the sole neurologic manifestation of poliovirus infection in the CNS of TgPVR21 mice is motor neuron death (38). Therefore, and because of the large number of animals enrolled in our assay, we did not evaluate the spinal cords of infected TgPVR21 mice histopathologically.

Athymic rat model of neoplastic meningitis. Subarachnoid catheters were implanted into female athymic rats using an established procedure described previously (39). Briefly, the rats (BIG:NIMRtm; 190-260 g) were anesthetized by an i.p. injection of ketamine (55 mg/mL) and xylazine (9 mg/mL) and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) with their necks flexed at a 70-degree angle using the tilt adapter. A midline sagittal incision was made from theinion to the laminar arch of C1, atlanto-occipital membrane was exposed and incised using a 20-gauge needle, and the underlying cisterna magna dura matter was opened. A PE-10 catheter (Intramedic, Franklin Lakes, NJ) with a 5-0 stainless steel wire stylet was then inserted into the subarachnoid space to the lumbar region (8.5 cm) by passing it along the posterior aspect of the spinal cord. The stylet was removed, a loose knot was tied in the catheter just above the opening of the dura, and the knot was secured in place with dental epoxy (Lang Dental Manufacturing Co., Chicago, IL). The exposed catheter end was then passed through the skin lateral to the incision. The incision was closed in three layers using 6-0 Ethilon (Ethicon, Somerville, NJ), and the catheter was temporarily occluded with 2-0 stainless steel wire. The rats were allowed to recover at least 7 days, and only rats showing normal weight and neurologic function and no evidence of infection were used in the experiments.

Neoplastic meningitis was induced by injecting tumor cells through the indwelling subarachnoid catheter. After anesthetizing rats with isoflurane, the wire stylet was removed, and 5.0×10^6 U87MGΔEGFR cells were injected in 40 µL of PBS using a 1,000-µL Hamilton syringe and injector (Hamilton Co., Reno, NV). The catheter was then flushed with 20 µL of PBS and reoccluded with 2-0 wire.

Toxicity and efficacy studies in athymic rats. For intrathecal toxicity studies, athymic rats were injected with 1.0×10^9 pfu PVS-RIPO in 60 µL of PBS. The weight, neurologic status, survival, and histologic examination of the neural axis and internal organs were compared with a control group treated with PBS. For efficacy studies, rats in the treatment groups were injected with 1.0×10^7 or 1.0×10^9 pfu PVS-RIPO in 60 µL of PBS, and rats in the control groups were injected with 1.0×10^9 pfu UV-inactivated PVS-RIPO in the same volume of PBS or

Table 1. Clinical score frequencies of a serotype 1 PV reference strain (WHO/I) and two lots of PVS-RIPO (0402018, 2nd passage; 0404007, 5th passage) in CD155 transgenic mice (TgPVR21)

Virus strain	Dose	Sex	Clinical score				Paralysis rate	Death rate
			0	1	2	n		
Reference (WHO/I)	1.75 log ₁₀ TCID ₅₀	F	11	5	5	16	0.312	0.312
		M	11	5	5	16	0.312	0.312
		Total	22	10	10	32	0.312	0.312
	2.75 log ₁₀ TCID ₅₀	F	5	11	11	16	0.687	0.687
		M	2	14	14	16	0.875	0.875
		Total	7	25	25	32	0.781	0.781
PVS-RIPO (L0402018)	1.75 log ₁₀ TCID ₅₀	F	16	0	0	16	0	0
		M	16	0	0	16	0	0
		Total	32	0	0	32	0	0
	2.75 log ₁₀ TCID ₅₀	F	16	0	0	16	0	0
		M	16	0	0	16	0	0
		Total	32	0	0	32	0	0
PVS-RIPO (L0404007)	1.75 log ₁₀ TCID ₅₀	F	16	0	0	16	0	0
		M	16	0	0	16	0	0
		Total	32	0	0	32	0	0
	2.75 log ₁₀ TCID ₅₀	F	16	0	0	16	0	0
		M	16	0	0	16	0	0
		Total	32	0	0	32	0	0

NOTE: The procedures were carried out in accordance with WHO standard operating procedures. Mice were inoculated by the intraspinal route and observed for clinical symptoms for 14 days. The clinical status of each mouse was recorded. Clinical score: 0 = no neurological symptoms; 1 = paralysis; 2 = death. Abbreviations: M, male; F, female.

just a PBS. Clinical neurologic function included testing of the stepping and placing reflex and the ability to climb a 60-degree incline ramp. Histologic examination was conducted on six representative cross-sections of the CNS, including the forebrain at level of lateral ventricles, hindbrain at the level of occipital lobe, and four equally spaced spinal sections, including the cauda equina. These sections were evaluated microscopically for hemorrhage, necrosis, edema, demyelination, and arachnoid fibrosis.

Statistical analysis. Survival estimates and median survival was determined by using the method of Kaplan and Meier (40). Survival data were compared using the log-rank test.

Results

Intrathecal toxicity in CD155-expressing transgenic mice. Because rodents do not express the human poliovirus receptor, CD155, we initially evaluated the intrathecal toxicity of PVS-RIPO in CD155 transgenic mice (TgPVR21). WHO/I and two clinical lots of PVS-RIPO (0402018, 2nd passage; 0404007, 5th passage in Vero cells) were administered intraspinally, and neurologic symptoms were evaluated from days 0 to 14 after the PVS-RIPO inoculation. In this toxicity study, two different

concentrations of virus (1.75 log₁₀ TCD₅₀ and 2.75 log₁₀ TCD) were tested. In the mice inoculated with low-titer WHO/I, 31.2% of female mice (5 of 16) and 31.2% of male mice (5 of 16) showed paralysis, and all symptomatic animals died as a result of virus infection (Table 1). Similarly, in mice inoculated with high-titer WHO/I, 68.7% of female mice (11 of 16) and 87.5% of male mice (14 of 16) showed paralysis and subsequent fatal involvement of respiratory musculature. However, in the low-titer and high-titer PVS-RIPO (0402018 and 0404007) inoculated groups, none of the mice developed neurologic symptoms, and all animals survived the experiment (Table 1).

Intrathecal toxicity in non-tumor-bearing rats. To evaluate any possible toxicity of PVS-RIPO after intrathecal delivery in athymic rats, we also injected 1.0 × 10⁹ plaque-forming units (pfu) of PVS-RIPO into the intrathecal space. At this dose, neither weight loss of >10% nor neurologic deficits were observed. Histologic examination of the neuroaxis revealed only mild pericatheter demyelination of spinal cord in both the PBS-treated group and in the PVS-RIPO-treated group (Table 2). This asymptomatic histologic finding is observed in all of studies

Table 2. Toxicologic analysis of intrathecal PVS-RIPO on athymic rats without tumor

Dose of PVS-RIPO injected	Survival	Neurologic symptoms	CNS			
			Hemorrhage	Necrosis	Demyelination	Arachnoid fibrosis
PBS	6/6	0/6	0/6	0/6	4/6	0/6
1.0 × 10 ⁹ pfu/60 μL	6/6	0/6	0/6	0/6	2/6	0/6

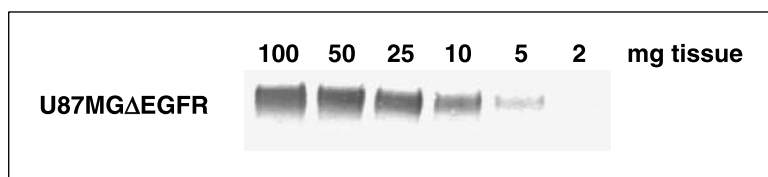


Fig. 1. Expression of the poliovirus receptor CD155 in human glioblastoma multiforme cell line (U87MGΔEGFR). Other glioblastoma multiforme cell lines and primary explants displayed a similar range of CD155 expression levels (28).

using this model and is likely due to catheter placement (13). No evidence of hemorrhage, necrosis, edema, or arachnoid fibrosis was identified in the brain or spinal cord of PVS-RIPO-treated rats. Additional histologic examination of internal organs, including heart, lung, liver, spleen, and kidney, of each rat showed no specific pathologic changes attributed to PVS-RIPO treatment.

U87MGΔEGFR^{III} expresses CD155 and is susceptible to PVS-RIPO. To evaluate the level of expression of the poliovirus receptor, CD155, in the human glioblastoma multiforme cell line U87MGΔEGFR, we used an antigen capture/Western blot assay. This assay showed that CD155 was detected in the U87MGΔEGFR cell line (Fig. 1). Next, we evaluated whether or not PVS-RIPO could be propagated in cultured U87MGΔEGFR cells and found that PVS-RIPO propagated at levels comparable with cell lines commonly used for large-scale propagation of virus in the laboratory (i.e., HeLa cells; ref. 32; Fig. 2). These results indicated that U87MGΔEGFR cells were appropriate targets for oncolytic PVS-RIPO therapy.

Efficacy of PVS-RIPO against neoplastic meningitis with a human glioblastoma multiforme xenograft. The therapeutic efficacy of intrathecal PVS-RIPO in an athymic rat model of human glioblastoma multiforme neoplastic meningitis induced by U87MGΔEGFR was investigated at two different doses, and survival was compared with groups of rats treated with PBS or UV light-inactivated PVS-RIPO. After inoculation of 5.0×10^6 U87MGΔEGFR cells, median survival was increased by 187.5% from 8 days in the PBS-treated group to 15 days in the group treated with 1.0×10^7 pfu PVS-RIPO ($P < 0.0001$; Fig. 3). Similarly, the median survival time was increased by 174.47% when compared with the group treated with the UV light-inactivated PVS-RIPO. In the group receiving the higher dose of PVS-RIPO (1.0×10^9 pfu), the median survival was increased by 200% compared with the PBS-treated group and 188.23% compared with the group treated with UV-inactivated PVS-RIPO ($P < 0.0001$). There was, however, no statistically significant dose-response relationship ($P = 0.345$). In addition to the increase in median survival, 1 of 10 rats in lower-dose PVS-RIPO-treated group and 3 of 10 rats in higher-dose PVS-RIPO-treated group survived over 60 days after tumor cell inoculation. Histologic examination of their brains and spinal cords showed no evidence of residual tumor.

Discussion

Regional delivery of therapeutic agents into the subarachnoid space for the treatment of neoplastic meningitis is an attractive strategy because it bypasses the blood-brain barrier and provides high concentrations of the therapeutic agent at the tumor site while minimizing systemic exposure and toxicity. Intrathecal delivery may be even more advantageous in the context of oncolytic virus therapy because it shields the virus from host immunity, allowing the virus to infect and spread more effectively within the tumor. Leptomeningeal involvement can

occur in up to 10% to 14% of high-grade gliomas. Nevertheless, the indications for intrathecal treatment remain extremely rare. However, as therapies improve for intraparenchymal disease, the incidence of treatable leptomeningeal spread may increase. The data presented here then provide proof of the principle that oncolytic viral therapy may be a viable option to investigate in these patients. We believe that this is important given the continued lack of chemotherapeutic agents available for this problem. In addition, the transgenic mouse data herein provide information on the potential unique toxicities of this approach in an immunocompetent model.

In our previously published work, we showed that the poliovirus receptor, CD155, is stably expressed in established glioblastoma multiforme cell lines as well as primary explant cultures (28). We have also previously shown that the oncolytic recombinant poliovirus, PVS-RIPO, is efficacious in an athymic rat model of intracerebral breast cancer metastasis (13). We now present new data based on our investigations with a clinical grade recombinant poliovirus, PVS-RIPO, prepared for human use by the NIH Rapid Access to Inventional Development program, that this oncolytic viral approach may also have efficacy in glioblastoma multiforme neoplastic meningitis after intrathecal delivery.

Although we show here that intrathecal PVS-RIPO therapy may be effective against human glioblastoma multiforme neoplastic meningitis in an athymic rat model, there may still be significant limitations to its success in humans that will need to be evaluated. For example, in our preclinical study, a

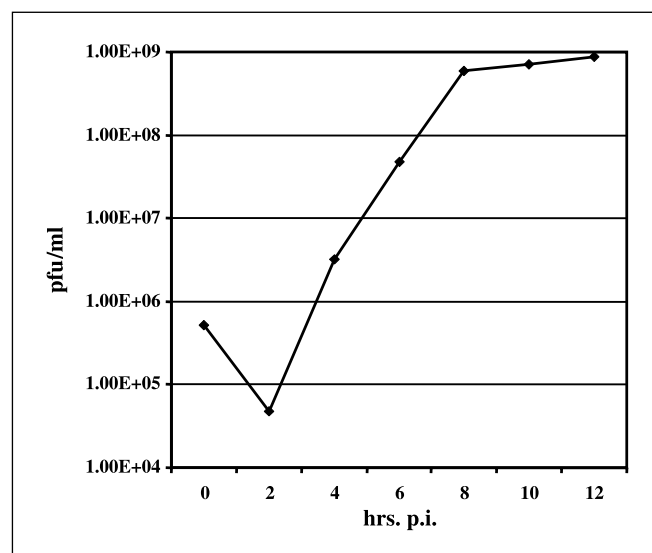
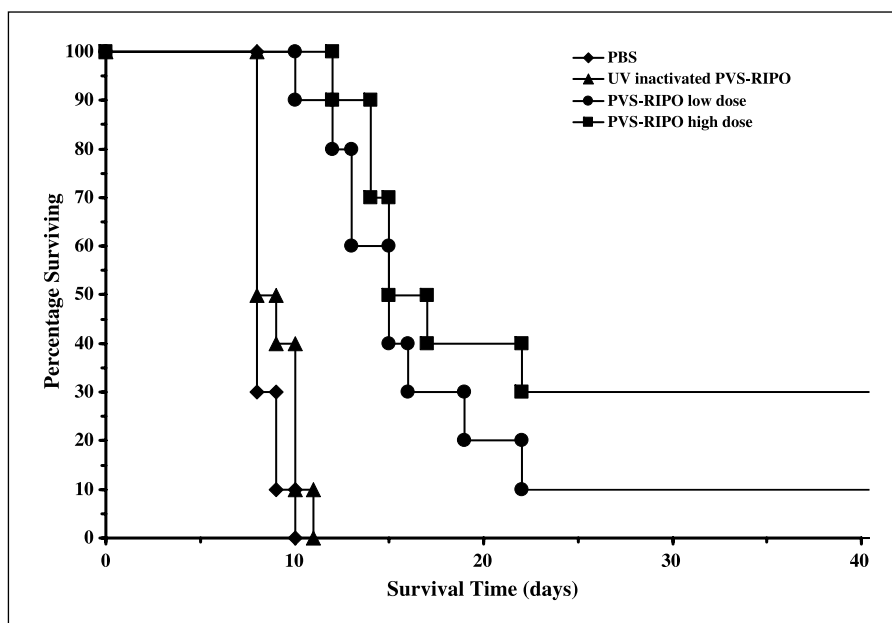


Fig. 2. Propagation of PVS-RIPO in human glioblastoma multiforme cell line, U87MGΔEGFR. One-step growth curves of PVS-RIPO were obtained after synchronized infection of monolayer cultures at a multiplicity of infection (*MOI*) of 10.

Fig. 3. Intrathecal treatment of human glioblastoma multiforme neoplastic meningitis with PVS-RIPO in an athymic rat model. Groups of athymic rats ($n = 10$) harboring intrathecal U87MG Δ EGFR xenografts were treated with PVS-RIPO administered at a dose of 1.0×10^7 pfu/40 μ L (●) or 1.0×10^9 pfu/40 μ L (■), 3 days after tumor cell inoculation. Control animals were treated with UV-inactivated PVS-RIPO (▲; 1.0×10^7 pfu) or PBS (◆).



two log increase in virus dose had no effect on the efficacy of intrathecal viral treatment. Although the lower dose of virus may be “saturating,” it remains possible that treatment failures are due to the existence of resistant subpopulations of cells, perhaps those not expressing CD155, or tumor locales not accessible to inoculated virus, notwithstanding the dose.

Another potential obstacle is the fact that all patients that might receive this therapy will have been previously vaccinated against poliovirus. However, available evidence suggests that neutralizing antibodies do not exist in the CNS of vaccinated individuals, and only patients who experience neurologic symptoms of poliovirus infection, indicating intraspinal replication of virus, produce neutralizing antibodies in the cerebrospinal fluid. Still, given the disruption of the blood-brain barrier in patients with glioblastoma multiforme, conclusive evidence for or against existing neutralizing antibodies within the CNS will need to await human studies.

Similarly, monkeys have been shown to be able to contract poliomyelitis after intracerebral administration of pathogenic poliovirus, even if they had been previously vaccinated, suggesting that prior vaccination does not limit viral spread

within the CNS (41). Although this is concerning for toxicity, with PVS-RIPO, our preclinical work has shown it to be devoid of neuropathogenic potential even in the complete absence of a preexisting immune response. This is partially due to the heterologous HRV2 internal ribosomal entry site that selectively abrogates viral replication in neuronal cells. Perhaps more important then, is the role that preexisting immunity may play in thwarting i.t. propagation of PVS-RIPO, and whether such a response will limit the efficacy of this approach.

Another potential concern with regard to human application is the potential for deleterious adaptation of the virus by mutation because all RNA viruses are inherently genetically unstable. All of our previous experiments have shown that adaptation to neurovirulence does not occur under the circumstances proposed for our clinical trials (13, 24). Nonetheless, such events will need to be monitored carefully during clinical trials as they can never be categorically excluded with replicating RNA viruses.

In conclusion, intrathecal delivery of PVS-RIPO seems to have promise as a safe and efficacious agent for human glioblastoma multiforme neoplastic meningitis and should be investigated in phase I clinical trials.

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