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In vivo biodistribution of a highly attenuated recombinant vesicular stomatitis virus expressing HIV-1 Gag following intramuscular, intranasal, or intravenous inoculation

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ABSTRACT

Recombinant vesicular stomatitis viruses (rVSVs) are being developed as potential HIV-1 vaccine candidates. To characterize the in vivo replication and dissemination of rVSV vectors in mice, high doses of a highly attenuated vector expressing HIV-1 Gag, rVSVIN-N4CT9-Gag1, and a prototypic reference virus, rVSVIN-HVVGag5, were delivered intramuscularly (IM), intranasally (IN), or intravenously (IV). We used quantitative, real-time RT-PCR (Q-PCR) and standard plaque assays to measure the temporal dissemination of these viruses to various tissues. Following IM inoculation, both viruses were detected primarily at the injection site as well as in draining lymph nodes; neither virus induced significant weight loss, pathologic signs, or evidence of neuroinvasion. In contrast, following IN inoculation, the prototypic virus was detected in all tissues tested and caused significant weight loss leading to death. IN administration of rVSVIN-N4CT9-Gag1 resulted in detection in numerous tissues (brain, lung, nasal turbinates, and lymph nodes) albeit in significantly reduced levels, which caused little or no weight loss nor any mortality. Following IV inoculation, both prototypic and attenuated viruses were detected by Q-PCR in all tissues tested. In contrast to the prototype, rVSVIN-N4CT9-Gag1 viral loads were significantly lower in all organs tested, and no infectious virus was detected in the brain following IV inoculation, despite the presence of viral RNA. These studies demonstrated significant differences in the biodistribution patterns of and the associated pathogenicity engendered by the prototypic and attenuated vectors in a highly susceptible host.

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1. Introduction

Vesicular stomatitis virus (VSV) is a prototypic virus of the Rhabdoviridae family, belonging to the order Nidovirales, which encompasses single stranded, nonsegmented, negative-sense RNA viruses with highly conserved gene order [1]. The two most common serotypes of VSV in the western hemisphere are designated as Indiana (VSVIN) and New Jersey (VSVNJ). In nature, VSV infects livestock causing a self-limiting disease. Although naturally occurring human infections with VSV are rare, VSV infections have been reported for individuals directly exposed to infected livestock, within laboratory environments, and living within endemic regions [2–11]. VSV infection of humans is typically either asymptomatic or causes a mild influenza-like illness [6]. Among small mammals, mice are readily infected experimentally via a variety of inoculation routes and thus have served as a tractable model for immunogenicity, pathogenicity, neurotropism and neurovirulence studies [12–20].

VSV has shown promise as a vaccine vector for a wide variety of human pathogens including HIV, papilloma virus, respiratory syncytial virus, SARS coronavirus, Ebola virus, Marburg virus, herpes simplex virus, Lassa fever virus, influenza virus [for review, see Ref. [21]], and hepatitis C virus [22]. Most of these studies were conducted with prototypic, recombinant VSV (rVSV) vectors that were derived from tissue culture-passaged, wild type (wt) VSV strains, which were shown to be somewhat attenuated when compared to the wt VSV [23]. More recently, in a non-human primate model for neurovirulence, we showed that the prototypic rVSV vectors caused a significant level of neuropathology following direct intrathalamic inoculation.
injection, albeit at reduced levels compared to the wild type virus [24]. These observations led to the conclusion that the prototypic rVSV vectors required further attenuation and preclinical analysis prior to clinical evaluation.

In the process of generating rVSVs with reduced vector pathogenicity, we developed a highly attenuated rVSV vector expressing the HIV-1 Gag gene, rVSVIN-N4CT9-Gag1, which was made by combining three attenuating approaches: insertion of HIV-1 Gag gene in the first position of the genome, shuffling of VSV N gene to the fourth position (N4) and use of a VSV G with its cytoplasmic tail truncated from 29 to 9 amino acids (CT9) [25–27]. Because there is an established gradient of transcription for VSV in which genes proximal to the single 3’ genomic promoter are expressed to the highest levels with a decrease in transcription for each gene as its distance from this promoter increases [28–33], we anticipated high levels of Gag expression, a concomitant reduction in VSV N expression, and a high degree of attenuation for the rVSVIN-N4CT9-Gag1 vector as compared to the prototypic rVSV. Indeed, the in vivo attenuation of rVSVIN-N4CT9-Gag1 was characterized by a dramatic rise in the mouse LD50 following intracranial (IC) inoculation [34]. However, this vector retained the ability to grow in vitro to high titers, and was capable of inducing immune responses in mice and macaques that were comparable to those obtained with the prototypic vector, rVSV-HIVGag5 [34], and unpublished observations).

An important aspect of characterizing a potential vaccine vector is to determine its pathogenicity and tissue distribution following in vivo administration. Notably, there is no experience with administration of rVSV vectors to humans, and when human infection with VSV does occur, it is not known which tissues are affected nor how the infection spreads. Therefore, we assessed the in vivo distribution and replication of prototypic (rVSVIN-HIVGag5) and attenuated (rVSVIN-N4CT9-Gag1) rVSV vectors in mice following three different routes of administration: intramuscular (IM), intranasal (IN), and intravenous (IV). The IM and IN routes were chosen because they have been considered for clinical evaluation. Furthermore, we tested the IV route to evaluate the effects of widespread dissemination of the viruses [35]. As an increased stringency parameter, we used a high dose of rVSV vectors in these studies, 10⁸ plaque forming units (PFU) per animal.

2. Materials and methods

2.1. Mouse

All animal care and procedures conformed to Institutional Animal Care and Use Committee guidelines. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Six- to seven-week old, inbred female BALB/c mice were obtained from Taconic Farms (Germantown, NY) and were acclimated for 1 week prior to inoculation. Transponders (BioMedic Data Systems Inc., Rockville, MD) used to identify mice and to aid in recording body weights and temperatures were inserted subcutaneously into the backs of mice as per the manufacturer’s instructions. During the course of these studies mice were closely monitored for signs of neurological disease such as paralysis or seizures. Any mice that exhibited signs of physical disability such as inactivity to stimulation, seizures, or complete paralysis were euthanized.

2.2. Viruses

The rVSVIN-HIVGag5 and the rVSVIN-N4CT9-Gag1 viruses have been previously described [24,34]. To prepare viral stocks for inoculating mice, baby hamster kidney cells (BHKs) were infected with each virus at a multiplicity of infection (MOI) between 0.1 and 1.0. The rVSVIN-HIVGag5 virus was harvested after 24 h at 37°C, and the rVSVIN-N4CT9-Gag1 virus was harvested after 48 h at 32°C. The harvested culture media was first subjected to a low-speed centrifugation (1500 rpm, 5 min, 4°C in a Beckman GS-6KR Refrigerated Centrifuge), and the supernatants were layered onto 10% sucrose cushions. Following ultracentrifugation (28,000 rpm, 80 min, 4°C in a Beckman SW28 rotor), media and sucrose were discarded and viral pellets were resuspended in phosphate buffered saline (PBS, Mediatech). Aliquots of reconstituted viruses were frozen in dry ice/ethanol baths and stored frozen at −80°C. To determine potency of virus stocks, an aliquot of each was thawed and titered on Vero cells in duplicate according to the procedure below.

2.3. Inoculations and clinical observations

Immediately prior to animal inoculations, frozen virus stocks were rapidly thawed at 37°C for 3–5 min, briefly mixed in a vortexer, and placed immediately on ice. Based on the concentration, each virus was diluted in PBS to a concentration of 10⁶ PFU/dose. PBS was used as a negative control for each group of mice. For IM and IN inoculations, mice were anesthetized with an IP injection of Ketamine (105 mg/kg) and Xylazine (10 mg/kg) diluted with sterile saline. For IM inoculations, each mouse was given one 50-µL injection in the right calf muscle. For IN inoculations, each mouse was given two 5-µl doses in the nose with a pause between inoculations to ensure proper uptake in nasal passages. For IV inoculations, mice were carefully warmed with a heat lamp and each mouse was given one 200-µL injection into a lateral tail vein. The health status of all mice was recorded for the study duration; in the day 24 groups of mice, weights and body temperatures were recorded daily for 2 weeks. Mice becoming bilaterally paralyzed or showing signs of distress or severe illness were euthanized and recorded as succumbing to VSV disease.

2.4. Tissue harvest and preparation

Tissues were harvested after transcardial perfusion with saline of the animal to remove any blood-associated VSV from the harvested organs (perfusion was not performed on mice receiving VSV IM, as previous studies showed that there is no VSV viremia following inoculation by this route). Mice were either euthanized by inhalation of CO₂ (for IM study) or anesthetized as described above and perfused (for IV and IN studies). In the latter case, death was achieved by the exsanguination caused by the perfusion. For perfusion, animals were placed in dorsal recumbency, the abdomen was wet with alcohol, and an incision was then made at the abdominal midline. The thoracic cavity was opened exposing the heart and lungs. A 20–21 gauge needle was then attached to a fluid bag containing PBS with an IV set. The needle was put into the apex of the left ventricle. The fluid line was then opened fully. The right auricle was then snipped. Sufficient fluid (maximum 100 mL) was then allowed to pump through the vasculature to evacuate all blood from the animal. The following organs were harvested from mice and tested for the presence of VSV RNA or virus: brain, liver, lung, kidney, spleen, muscle (corresponding to injection site for IM route, in the IM and IV studies only), nasal turbinates (IN studies only) and draining lymph nodes (popliteal, inguinal, and iliac for IM; and cervical for IN). Blood was also collected at the time of organ harvest, prior to perfusion. Each piece of tissue was weighed, suspended in 4°C phosphate-buffered saline (PBS)/sucrose phosphate buffer with glutamine (SPBG, 0.2 M sucrose, 7.0 mM K2HPO4, 3.8 mM KH2PO4, 5.0 mM glutamic acid) to 10% weight/volume (w/v), and homogenized using an Omni TM Tissue Homogenizing Kit with autoclavable probes. Lymph nodes and nasal turbinates were resuspended directly in 2.0 mL PBS/SPBG, producing an approximate 10% (w/v) suspension. For determination of viral loads, 140 µL aliquots...
were removed and stored at −70 °C for RNA extraction and virus titration.

2.5. Quantitative real-time RT-PCR

Viral nucleic acid was extracted from homogenized tissues using the RNeasy Mini kit (Qiagen, Valencia, CA) and was reverse transcribed into cDNA using methods previously described [36]. Briefly, detection of viral genomic RNA (negative sense) was done in a 20-μL reaction mix containing total RNA from tissue samples (5.0 μL or 0.5 μg), VSV N and the large ribosomal protein L15 housekeeping gene (RPLO) forward primers, and reagents from the Sensiscript Reverse Transcriptase kit (Qiagen). To detect mRNA transcripts, 10 μM of the anchored oligo-dT primer was substituted for the VSV N specific-forward primer. Otherwise, the reagents and the conditions for the reverse transcription step remained as described. Primer and probe sets for detecting RNA encoding VSV N and HIV Gag and the primer nucleotide sequence for the anchored oligo-dT primer have been previously described [36]. The primer-probe set RPLO was selected using the Primer Express software version 2.0 (Applied Biosystems, Foster, CA) and contained the following sequences: forward primer, 5′-GCCAGCTCAGAACAATCTGTCATG-3′ (anneals between residues 443 and 465); reverse primer, 5′-ATGCCAAAGGCTGGA-3′ (anneals between residues 484 and 502); TaqMan probe, 5′-CAGAGACCTCCCT-3′ (anneals between residues 469 and 482). The duplex quantitative real-time PCR to detect genomic RNA was carried out using the Quantitect Multiplex PCR Kit (Qiagen). A 30-μL PCR reaction containing 5 μL of each 10-fold dilution of the VSV N, HIV Gag, and RPLO cDNA used to generate standard curves or 5 μL of cDNA from tissue samples, 15 μL of 2 × Quantitect Multiplex PCR Master Mix, 400 nM each of the forward and reverse primers for VSV N and RPLO, and 200 nM of each probe. For the duplex assay to detect mRNA from cDNA, the reaction mix remained the same except HIV Gag and RPLO forward and reverse primers and probes were used. In order to distinguish the amplicons, the probes for VSV-N were labeled with FAM (6-carboxyfluorescein), HIV-Gag with VIC (6-carboxyrhodamine 6G), and RPLO with NED. Amplification and detection were performed with an ABI Prism 7500 Fast Real-Time PCR System using the following conditions: 15 min at 95 °C to activate the HotStar Taq DNA polymerase, followed by 40 cycles of 60 s at 94 °C and 90 s at 60 °C. All samples were tested in duplicate. For calculating group means, the detection limit was used for any sample that was below the limit of detection of the assay (0.5 PFU/10 mg of tissue or 50 PFU/mL of blood).

2.6. Virus titration

Briefly, the titer of virus was determined by performing 10-fold serial dilutions of samples in DMEM supplemented with 2% FBS. One-tenth the volume of each serial dilution (0.1 mL) was used to infect confluent Vero cell monolayers in 6-well plates (each sample infected in duplicate wells). After adding 0.4 mL of DMEM/2% FBS per well, infected cells were incubated at room temperature (RT) for 15 min followed by 37 °C for 30 min (in 5% CO2 incubator), the inoculum was removed from each well and replaced with 3.0 mL of 1% Sea Plaque agarose (Cambrex) in DMEM/2% FBS overlay. The plates were incubated in a 32 or 37 °C incubator until plaque formation (24–48 h). Agarose plugs were carefully removed with a small spatula, and the monolayers were fixed with 1:1 methanol:acetic acid mixture (2.0 mL per well) at RT for 30 min. After washing with PBS, 1.5 mL of blocking solution (5% milk in 0.1% Tween-20 Tris Buffered Saline [TTBS]) was added to each well and incubated at RT for 30–60 min. Following PBS wash, the monolayers were incubated at RT for 1 h with 1.0 mL per well of primary antibodies (1:1 mixture if mAbs 1-1 and 1-14 [37] diluted 1:2000 in TTBS). Monolayers were then washed with PBS and incubated at RT for 30–60 min with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG [Biorad] diluted 1:2000 in TTBS). Following PBS wash, the monolayers were incubated at RT for 5–10 min with 1.0 mL per well of DAB substrate (one 3,3′-Diaminobenzidine Tablet [Sigma] in 20 mL PBS and 7 μL H2O2). After counting the stained plaques, the virus titer was calculated as mean PFU/10 mg of homogenized tissue or PFU/mL of blood. For calculating group means, the detection limit was used for any sample that was below the limit of detection of the assay (0.5 PFU/10 mg of tissue or 50 PFU/mL of blood).

2.7. Statistical analyses

Data are expressed as mean ± SEM of five mice per test group. Where appropriate, statistical comparisons were conducted on log-transformed data using two-tailed unpaired t tests. In cases where all observations in one of the groups were below the limits of detection, Fisher’s exact test was used. A value of p < 0.05 was considered statistically significant and the degree of significance is represented in figures by: *p < 0.05; **p < 0.001; ***p < 0.0001.

2.8. Histopathology

Whole brains were fixed in 10% formalin immediately following necropsy. For each mouse, transverse, H&E-stained tissue sections of the olfactory lobes (OL), the cerebrum at the level of the corpus callosum, the cerebrum at the level of the optic chiasm, and the cerebellum with medulla oblongata at the level of the parafocculus were examined by a board certified veterinary pathologist. Each section was evaluated for the following diagnostic criteria: inflammation, perivascular (cuffing); inflammation/degeneration, neuronal/neurophil (gliosis); inflammation/degeneration, ventricular/periventricular; inflammation, meninges; degeneration, axonal or myelin; vacuolation, neuropil.

For each of the diagnostic criteria, a subjective severity score was assigned, using a scale where: 0 = normal, change not present; 1 = change present, minimal severity; 2 = change present, mild severity; 3 = change present, moderate severity; 4 = change present, marked severity; 5 = change present, severe.

3. Results

Previous studies have shown that a highly attenuated vector, rSVSN-IN4CT9-Gag1 [hereafter referred to as N4CT9-Gag1], had greatly reduced neurovirulence yet retained potent immunogenicity in mice compared to the prototypic vector, rSVSN-IN/HV Gag5, hereafter referred to as Gag5 [34]. To better understand virus dissemination following vaccination, we performed a series of studies comparing the tissue distribution of these two vectors in mice, following high dose (108 PFU) administration by the IM, IN, or IV routes. The initial study design included tissue harvests on days 0 (4 h post-inoculation), 1, 2, 4, and 8 post-inoculation for viral load assessment; however, because of persistent viral RNA in some tissues at day 8 in the IM study, additional later time points were collected in both the IN and IV studies.

3.1. Pathologic parameters following IM inoculations

Mice were inoculated by the IM route with Gag5, N4CT9-Gag1, or PBS as a control and monitored clinically for 2 weeks. Mice in the Gag5 group lost an average of 5% of their body weight on day 1 after inoculation and returned to normal weight by day 3, after which their weights matched the trend observed for the PBS control mice (Fig. 1A). There were no significant differences in body temperatures between the Gag5 group and the PBS group (data not shown), nor any clinical signs such as matted fur, paralysis, or death
Fig. 1. Mouse body weights and viral loads following IM inoculation. (A and B) Average body weights expressed as the percent change from day 0 weights versus days post-inoculation. (C and D) Average Gag mRNA copies per 10 mg of the indicated tissue or per mL of blood as determined by Q-PCR. (E and F) Average gRNA copies per 10 mg of the indicated tissues or per mL of blood as determined by Q-PCR. (G and H) Average infectious virus in plaque forming units (PFU) per 10 mg of tissue or per mL of blood as determined by standard plaque assay. (A), (C), (E), and (G): mice inoculated with rVSVIN-HIVGag5. (B), (D), (F), and (H): mice inoculated with rVSVIN-N4CT9-Gag1. Dotted lines indicate limits of detection of the assays. Limit of detection for Q-PCR = 5 × 10^3 copies/10 mg of tissue or 4 × 10^4 copies/mL of blood. Limit of detection of the plaque assay = 0.5 PFU/10 mg of tissue or 50 PFU/mL of blood. Bars at each time point indicate standard error. For comparison of rVSVIN-HIVGag5 to rVSVIN-N4CT9-Gag1, *p < 0.05; **p < 0.001; ***p < 0.0001.

Table 1
Clinical signs of disease in mice following inoculation.

| Virus          | Route | Number of mice immunized | Mattred Fur | Paralysis | Euthanized | Found Dead |
|---------------|-------|--------------------------|-------------|-----------|------------|------------|
| rVSVIN-HIVGag5| IM    | 35                       | 0 (0%)      | 0 (0%)    | 0 (0%)     | 0 (0%)     |
|               | IV    | 50                       | 0 (0%)      | 50 (100%) | 12 (24%)   | 0 (0%)     |
|               | IN    | 90                       | 0 (0%)      | 50 (100%) | 16 (18%)   | 6 (6.7%)   |
| rVSVIN-N4CT9-Gag1| IM    | 35                       | 0 (0%)      | 0 (0%)    | 0 (0%)     | 0 (0%)     |
|                | IV    | 50                       | 0 (0%)      | 0 (0%)    | 0 (0%)     | 0 (0%)     |
|                | IN    | 50                       | 0 (0%)      | 0 (0%)    | 0 (0%)     | 0 (0%)     |
3.3. Pathologic parameters following IM inoculations

Mice inoculated by the IN route with either Gag5, N4CT9-Gag1, or PBS as a control were monitored clinically for 2 weeks. Mice in the Gag5 group lost weight steadily until days 5–6, at which time they had lost an average of 25% of their initial body weights (Fig. 2A); all of the mice appeared ill (Table 1). Mice that did not die nor required euthanasia began to gain weight following day 7, but never recovered to the day 0 levels. Although the average body temperatures in these mice were similar to the PBS control group throughout the study (data not shown), 100% of these mice in the Gag5 group had matted fur, 9% developed paralysis, and a total of 16% died by day 10. In contrast, mice in the N4CT9-Gag1 group were similar to the PBS control group throughout the study (data not shown), 100% of these mice in the Gag5 group had matted fur, 9% developed paralysis, and a total of 16% died by day 10. Mice that were inoculated with N4CT9-Gag1 by the IN route had detectable Gag mRNA, gRNA, and infectious virus in the following tissues: brain, lung, nasal turbinates, and lymph nodes (Fig. 2D, F, and H). Only one mouse had both Gag mRNA and gRNA copies just above the detection limit on day 2 in the spleen. In addition, one mouse had detectable gRNA in the liver at day 0 (Fig. 2F). As in the Gag5 group, N4CT9-Gag1 Gag mRNA and gRNA persisted the longest in brain (day 17), nasal turbinates (day 17, with one mouse having gRNA just above the detection limit at day 31), and lymph nodes (day 31). Infectious virus was cleared from the lung by day 1 and the lymph nodes by day 4, but did not reach undetectable levels in nasal turbinates or brain until day 8. In most tissues at most time points, the N4CT9-Gag1 viral loads were significantly lower than those of the Gag5 virus. Overall, there were more significant differences in gRNA levels (Fig. 2F) for these two viruses than were observed for Gag mRNA. Finally, infectious virus was significantly lower in mice treated with N4CT9-Gag1 when compared to the Gag5 group in all tissues tested, excluding blood, where N4CT9-Gag1 was undetectable at all time points tested. Of particular note in the N4CT9-Gag1 group, peak infectious titers and gRNA levels detected in brain tissues (day 2) were 100-fold and 500-fold lower, respectively, than the Gag5 group.

3.4. Viral loads following IN inoculations

In mice inoculated with Gag5 by the IN route, viral mRNA and gRNA were detected in all tissues tested (Fig. 2E and C, respectively). As in the IM study, Gag mRNA, gRNA, and infectious virus were detected at the highest levels at the site of inoculation, in this case the nasal turbinates. While overall viral loads varied among the different tissues, the Gag mRNA copies, gRNA copies, and titers of infectious virus displayed similar kinetics in each tissue type; furthermore, Gag mRNA and gRNA copies were nearly equivalent in most tissues. Based on the tissue distribution at day 0, the inoculum appeared to be contained in the nasal turbinates and lungs; however, a small number of viral genomes (but no Gag mRNA) were detected in the brain tissue of mice on day 0, which presumably corresponded to input virus. Gag mRNA and/or gRNA persisted the longest in brain (day 17), nasal turbinates and lymph nodes (both day 34, with one mouse for each tissue having Gag mRNA just above the detection limit at day 41). Infectious virus was cleared from all tissues by day 4 or 8, except for brain tissue, where it was cleared by day 10.

Mice that were inoculated with N4CT9-Gag1 by the IN route had detectable Gag mRNA, gRNA, and/or infectious virus in the following tissues: brain, lung, nasal turbinates, and lymph nodes (Fig. 2D, F, and H). Only one mouse had both Gag mRNA and gRNA copies just above the detection limit on day 2 in the spleen. In addition, one mouse had detectable gRNA in the liver at day 0 (Fig. 2F). As in the Gag5 group, N4CT9-Gag1 Gag mRNA and gRNA persisted the longest in brain (day 17), nasal turbinates (day 17, with one mouse having gRNA just above the detection limit at day 31), and lymph nodes (day 31). Infectious virus was cleared from the lung by day 1 and the lymph nodes by day 4, but did not reach undetectable levels in nasal turbinates or brain until day 8. In most tissues at most time points, the N4CT9-Gag1 viral loads were significantly lower than those of the Gag5 virus. Overall, there were more significant differences in gRNA levels (Fig. 2F) for these two viruses than were observed for Gag mRNA. Finally, infectious virus was significantly lower in mice treated with N4CT9-Gag1 when compared to the Gag5 group in all tissues tested, excluding blood, where N4CT9-Gag1 was undetectable at all time points tested. Of particular note in the N4CT9-Gag1 group, peak infectious titers and gRNA levels detected in brain tissues (day 2) were 100-fold and 500-fold lower, respectively, than the Gag5 group.

3.5. Histopathology following IN inoculations

Fixed brains were sectioned, examined, and scored as described in Materials and Methods (Table 2). One mouse inoculated with Gag5 virus by the IN route exhibited mild inflammation with minimal neurophil vacuolation at day 8 in two sections examined. At day 24, focal, minimal perivascular inflammation was present in one section examined from one of six mice. Microscopic changes were not observed in any other sections examined from Gag5-inoculated mice or in any sections from either the N4CT9-Gag1 or PBS groups at days 8 or 24.

3.6. Pathologic parameters following IV inoculations

High dose IV inoculations of mice with Gag5, N4CT9-Gag1, or PBS as a control were included as a stringent means of analyzing vector-associated pathogenicity and widespread dissemination. In the IV Gag5 group, mice lost 10% of their initial body weights on days 1–2 and slowly regained weight over the course of the study (Fig. 3A). Although there were no differences in body temperatures compared to the PBS control group (data not shown), 100% of these mice had matted fur and 4% became paralyzed, requiring euthanasia (Table 1). Mice inoculated with N4CT9-Gag1 by the IV route lost 5% of their body weight on day 1, but quickly returned to normal weights by day 2 (Fig. 3B). Again, there were no differences in body temperatures compared to the PBS group (data not shown). Additionally, no clinical signs were observed in any of the mice (Table 1).
3.7. Viral loads following IV inoculations

Following IV inoculation with Gag5, virus was detected in all tissues tested (Fig. 3C, E, and G), particularly in the Q-PCR assay. In the blood, viral loads peaked on days 0–1 and were cleared between days 2 and 4. The highest viral loads were detected in the spleen, in which virus was cleared by day 4 but gRNA remained detectable through day 10. In liver, lung, kidney, and muscle, viral RNA peaked between days 0–2 and was at or near the detection limit by day 8; furthermore, infectious virus was either undetectable in these tissues (lung and kidney) or was cleared by day 4 (liver and muscle). Viral loads in the lymph nodes were found to be highest on days 0–2 and persisted through day 4 (infectious virus), day 8 (Gag mRNA), or day 17 (gRNA), which was the last time point taken for this route. Finally, viral loads peaked in the brain at later time points than other tissues, and while infectious virus was cleared by day 8, Gag mRNA persisted through day 10 and gRNA persisted through day 17 in two out of five mice.

Viral loads were similarly detected in all tissues tested in the IV N4CT9-Gag1 group (Fig. 3D, F, and H). In this group, the highest viral loads were also observed in the spleen, in which infectious virus was cleared by day 4 and viral RNA, particularly genomic, persisted through day 10. In liver, lung, kidney, and muscle, viral RNA was detectable only on days 0–2 (with low levels of gRNA still detectable in liver tissue on day 4), and infectious virus was undetectable in all of these tissues except muscle (days 0–1). In the lymph node,
Fig. 3. Mouse body weights and viral loads following IV inoculation. (A and B) Average body weights expressed as the percent change from day 0 weights versus days post-inoculation. (C and D) Average Gag mRNA copies per 10 mg of the indicated tissue or per mL of blood as determined by Q-PCR. (E and F) Average gRNA copies per 10 mg of the indicated tissues or per mL of blood as determined by standard plaque assay. (G and H) Average infectious virus in plaque forming units (PFU) per 10 mg of tissue or per mL of blood as determined by standard plaque assay. Bars at each time point indicate standard error. For comparison of rVSVIN-HIVGag5 to rVSVIN-N4CT9-Gag1, *p < 0.05; **p < 0.001; ***p < 0.0001.

Table 2
Brain histopathology results.

| Virus          | Routea | Day | No. with lesions/no. in groupb | Observationsc |
|----------------|--------|-----|-------------------------------|---------------|
| rVSVIN-HIVGag5 | IN     | 8   | 1/5                           | Mild inflammation and minimal neurophil vacuolation |
|                | IV     | 24  | 1/6                           | Minimal perivascular inflammation |
| rVSVIN-N4CT9-Gag1 | IN | 8   | 0/5                           | Minimal to mild multifocal inflammation |
|                | IV     | 24  | 0/5                           | |

a The IM samples were not tested because there were no measurable viral loads in the brain tissue samples at any time point post-inoculation.
b Number of animals that had at least one lesion in one brain section compared to the number of animals in that group.
c All animals in PBS groups were negative at days 8 and 24 in both the IN and IV routes.
while infectious virus was cleared by day 2, viral RNA remained detectable through day 10 (Fig. 3H). In the brain, viral RNA peaked on day 4 and was undetectable by day 8. As in the IN study, the gRNA levels measured in the N4CT9-Gag1 group were significantly lower than in the Gag5 group at more time points than the Gag mRNA levels, although the latter still demonstrated significant differences between these two groups (Fig. 3D and F). Additionally, in each tissue and time point that infectious N4CT9-Gag1 virus was detected, the levels were significantly lower than the Gag5 virus (Fig. 3H) in the corresponding tissues.

3.8. Histopathology following IV inoculations

For mice inoculated IV with Gag5 virus, minimal to mild multifocal, nonsuppurative inflammation was observed in three of five mice in the day 8 samples, and no changes were observed in the brains of day 24 mice (Table 2). No changes were present in the brains of the N4CT9-Gag1 groups or the PBS control mice at either day 8 or 24.

4. Discussion

While mice are not considered a natural host for VSV, they have been used as an animal model for studying VSV pathogenesis for over 70 years [38]. The exquisite sensitivity of mice to VSV infection has proven them as a valuable research tool for assessing virulence of wild type and attenuated strains of VSV [12–20]. While the neurovirulence associated with VSV infection in mice has been well studied, there has not been much advancement in the understanding of the biodistribution of VSV in mice since the pioneering work of Sabin and Olitsky in the 1930s [39–43]. Presently, we used highly sensitive Q-PCR assays to determine that for both the highly attenuated N4CT9-Gag1 and prototypic Gag5 viruses, infection was limited to the inoculation site and draining lymphoid organs following IM inoculation, while more widespread infection was detected when viruses were inoculated via the IN or IV route.

Recombinant VSV vectors are currently being developed as vaccine vectors for HIV and other pathogens, and as with any live viral vector, they must be sufficiently attenuated to be administered to humans. The attenuated rVSV tested in this study was shown to be well tolerated at high doses in the highly sensitive mouse model by all three routes of inoculation whereas the more virulent prototypic VSV induced significant illness following IN or IV inoculation. Both the attenuated and prototypic viruses showed minimal dissemination following IM inoculation, supporting the selection of this route for future first-in-man clinical trials with rVSV vectors. In addition, the attenuated rVSV did not exhibit any adverse clinical signs such as weight loss, paralysis or death despite its widespread tissue distribution in the IV and IN studies. Consistent with its attenuated phenotype in vitro, the N4CT9-Gag1 virus did not replicate as vigorously in vivo as the prototypic Gag5 virus, which was underscored by the statistical significance of the reduced viral loads in the majority of tissues for the N4CT9-Gag1 groups as compared to the Gag5 groups at the majority of time points.

Furthermore, despite the significantly reduced replication levels of the attenuated virus compared to the prototype, the anti-Gag immune responses in mice inoculated IM with the attenuated virus were equal to or greater than those responses induced with the prototypic vector [34]. This robust immunogenicity associated with the use of the attenuated vector may be due to enhanced expression of the Gag gene by nature of its promoter-proximal position within the modified genome (Gag1), as compared to the prototypic vector, in which the Gag gene is more distal to the genomic promoter (Gag5). Indeed, this notion is supported in these studies by comparing the Gag mRNA levels to the gRNA levels between the two vectors. For all three routes of inoculation, the gRNA levels of the attenuated vector were significantly lower than the corresponding gRNA levels of the prototypic vector in more tissues and at more time points than was observed for the corresponding Gag mRNA levels (compare the number of statistically significant differences in panels D and F in Figs. 1–3).

The restricted spread of VSV following IM inoculation in adult mice, originally described by Sabin and Olitsky [41], was similar to what was recently reported for infection in cattle, a natural host [44]. In the bovine study, viral spread was limited to the inoculation site and draining lymph nodes in a similar manner to what was seen with IM inoculation in mice in this study. This limited spread in cattle by an experimental model of natural infection may explain why cattle naturally infected with VSV do not develop neurological disease. Similarly, wt VSVNJ demonstrated restricted ability to spread to neuronal tissue following intracranial inoculation of adult deer mice; this restriction was absent from juvenile deer mice, however [14]. In the murine studies described herein, neither the attenuated virus nor the more virulent prototypic virus yielded adverse neurological findings or evidence of neuroinvasion after IM inoculation. Sabin and Olitsky proposed that there are natural barriers to the spread of VSV in adult mice that keep VSV from entering neuronal tissue following IM inoculation [41], an idea supported by the present studies.

Interestingly, while VSV infection was detected in brains of animals infected IV with either attenuated or prototypic virus, there was minimal (Gag5) or no (N4CT9) detectable neuropathology observed in the brains, despite the kinetics of infection indicating active replication (peaking at days 2–4) in the brain. Moreover, the level of Gag5 detected in the brain was well above the doses required to induce lethal encephalitis following direct intracranial (IC) inoculation, as the IC LD50 for Gag5 is less than 5 PFU [25]. Studies utilizing VSV vectors expressing GFP are being pursued to determine the cellular localization of VSV infection in the brain and other organs following IV inoculation.

VSV administered by the IN route of inoculation is pathogenic in mice, especially in young mice. Juvenile mice succumb to a lethal encephalitis following transmission of VSV from the olfactory epithelium to the olfactory bulb via the olfactory nerve [14,45–49]. The results reported herein demonstrated that IN inoculation with the more virulent prototypic virus induced more weight loss and clinical signs of disease than was observed during systemic infection following IV inoculation. Following IN inoculation, Gag5 viral loads peaked at day 2 in brain. Since whole brain homogenates were used for virus analysis in this study, it is possible that the infectious virus or viral RNA may have been concentrated in the olfactory bulb region of the brain. The peak virus titer of ∼106 PFU/10 mg of tissue seen in brain homogenates of animals receiving prototypic rVSV by the IN route was >105 times higher than the IC LD50, yet the mice showed limited morbidity and minimal neuropathology. It is possible that more severe neuropathology occurred in the subset of mice that died following IN inoculation with the prototypic vector, but we were unable to assess their brains for viral loads or histopathology. In contrast, mice receiving attenuated rVSV intranasally exhibited significantly lower levels of infectious virus in brain and exhibited no signs of morbidity or mortality. Furthermore, there was less systemic spread of virus following IN instillation of the attenuated virus as well as no detectable viremia. These findings with N4CT9-Gag1 are in agreement with analysis of related attenuated VSV vectors that showed that compared to the wt vector, a truncated cytoplasmic tail VSV vector (rVSV-CT1) spread significantly less to the lymph nodes and other organs when inoculated by the IV route [50].

A few previous studies have addressed persistence of VSV following infection in cattle, its natural host [51] and in experimental rodent models [50–53]. In order to understand the nature of VSV persistence in animals following infection and convalescence,
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