Evaluation of metaphylactic RNA interference to prevent equine herpesvirus type 1 infection in experimental herpesvirus myeloencephalopathy in horses

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Objective—To evaluate metaphylactic RNA interference to prevent equine herpesvirus type 1 (EHV-1) infection in experimental herpesvirus myeloencephalopathy in horses and to determine whether horses infected with a neuropathogenic strain of the virus that develop equine herpesvirus myeloencephalopathy (EHM) have differences in viremia.

Animals—13 seronegative horses.

Procedures—EHV-1 strain Ab4 was administered intranasally on day 0, and small interfering RNAs (siRNAs [EHV-1 specific siRNAs \(n=7\) or an irrelevant siRNA \(6\)]) were administered intranasally 24 hours before and 12, 24, 36, and 48 hours after infection. Physical and neurologic examinations, nasal swab specimens, and blood samples were collected for virus isolation and quantitative PCR assay. Data from the study were combined with data from a previous study of 14 horses.

Results—No significant difference was detected in clinical variables, viremia, or detection of EHV-1 in nasal swab specimens of horses treated with the EHV-1 targeted siRNAs (sigB3-siOr2) versus controls. No significant differences in viremia were detected between horses that developed EHM and those that did not.

Conclusions and Clinical Relevance—Administration of siRNAs targeted against EHV-1 around the time of EHV-1 infection was not protective with this experimental design. Horses infected with the neuropathogenic EHV-1 strain Ab4 that developed EHM did not have a more pronounced viremia. (Am J Vet Res 2013;74:248–256)

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and environmental factors. Equine herpesvirus type 1 strain Ab4, originally isolated from a quadriplegic mare, is the prototypical neuropathogenic strain used to investigate the pathogenesis of EHM. For example, Ab4 induces viremia more efficiently than non-neuropathogenic strains under field conditions as well as in experimental infections, which lead to an increased and prolonged presence of virus in the bloodstream.3,8 The problem with experimentally induced EHV-1 disease, however, is that neurologic signs are induced inconsistently. This is most likely due to variations in the experimental methods, for example, the dose and strain of the virus used, the route of infection, and host factors (such as use of aged mares, pregnant mares, and ponies); all of these differences make comparisons among studies difficult.3,8–13 Despite many laboratories’ efforts, experimental studies of EHM do not reliably induce clinical EHM, and therefore, conclusions regarding vaccination efficacy or other interventions against EHM are problematic.

Prevention of EHV-1 infection is mainly limited to vaccination, but protection is incomplete.8,14 Still, commercially available inactivated and MLV EHV-1 vaccines can decrease clinical disease and virus shedding, the latter being an important factor for herd immunity.9 Treatment of EHV-1 infections generally relies on supportive therapy, and investigations of the efficacy of antiviral drugs (acyclovir and valacyclovir) have yielded conflicting results.15–18 Moreover, poor bioavailability, cost of treatment for an adult horse, and lack of demonstrated clinical efficacy have limited the use of these antiviral drugs in equine practice.6,16 Efficacy problems with vaccines and antiviral drugs have prompted searches for alternatives for the prophylaxis of EHV-1-associated diseases. For those reasons, RNA interference by use of siRNAs against essential viral genes in general (and in viral genes in herpesviruses, specifically) has recently gained interest.19–21

Previously, the authors’ laboratory generated siRNAs targeted against 2 essential EHV-1 genes. The sigB3 siRNA is targeted against EHV-1 genes encoding glycoprotein B required for viral entry and cell-to-cell transmission, and the siOri2 siRNA is targeted against EHV-1 genes encoding the origin binding protein helicase required for replication of the viral genome. Efficacy was demonstrated in vitro as well as in a model of EHV-1 infection in mice, by confirming that siRNA treatment in vivo significantly reduced viral replication and clinical signs.22 In a subsequent study, the efficacy of an intranasal application of these siRNAs was then tested in the natural host species (horse) infected with the neuropathogenic EHV-1 strain Ab4. No significant differences were found in the amount of EHV-1 nasal shedding or in the level or duration of viremia between treated horses and control horses. However, there was a significant difference in the proportion of horses that developed severe EHM and required euthanasia. Three of 4 horses administered an irrelevant siRNA (siLuc) developed EHM and had to be euthanized, whereas only 2 of 10 of the EHV-1 targeted siRNA (sigB3-siOri2)–treated horses developed EHM and those 2 survived.

The purpose of the study reported here was to evaluate metaphylactic RNA interference at higher doses and at more frequent intervals than previously tested to prevent EHV-1 infection in experimental herpesvirus myeloencephalopathy in horses and to determine whether horses infected with a neuropathogenic strain of the virus that develop EHM have differences in viremia. Our hypothesis was that horses with EHM have a higher magnitude and longer duration of viremia than those that do not develop neurologic signs.

Materials and Methods

Animals—The protocol for this study was approved by the Institutional Animal Care and Use Committee at Cornell University. The inclusion criteria were an EHV-1 SNT ≤ 64 and no spontaneous EHV-1 reactivation (as determined by the absence of an increase in SNT) during the 8-week observation period prior to the start of the experiment. Previous vaccination history of the horses was unknown. When the horses arrived from auction, they were dewormed with a moxidectin and praziquantal product and, 4 weeks later, with fenbendazole at label dosages. The horses were kept on pasture and fed grass hay ad libitum and sweet feed twice daily prior to the study. All horses were moved into a biosecurity level 2 isolation facility, randomly assigned (via numbers drawn from hat) to treatment (n = 8) and control (6) groups, and allowed to acclimate for 4 days. Investigators involved in the clinical evaluations and laboratory assays remained unaware of group assignments until all data were gathered and samples were fully processed.

Metaphylactic siRNA treatments and EHV-1 infection—The siRNAs used in this study have been described and target the EHV-1 gB (sigB3) and Ori-binding protein (siOri2).21 Horses in the control group received an irrelevant siRNA targeting firefly luciferase (siLuc). The siRNAs were reconstituted according to manufacturer’s instructions and diluted in PBS solution. No carrier molecules were used, nor were the siRNAs chemically modified.

Metaphylactic administration of siRNA was performed 24 hours prior and 12, 24, 36, and 48 hours after EHV-1 infection. Due to the number of intranasal treatments, only when deemed necessary were horses sedated with detomidine (0.01 mg/kg) prior to intranasal siRNA treatment or EHV-1 infection. Eight horses received a mixture of 5nM each of sigB3 and siOri2, intranasally delivered with a mucosal atomization device.6 Six control horses received an equal amount of the irrelevant siLuc (10nM), which was administered analogously to the treated group. The first dose of siRNA (~24 hours) was administered in a total volume of 6 mL; all subsequent doses were in a volume of 4 mL and were divided between the 2 nostrils.

All horses were infected intranasally with 1 × 10^7 PFU of EHV-1 strain Ab4 (day 0) by means of the same application technique as described for the siRNA. This EHV-1 strain possesses the D752 SNP and is typed as neuropathogenic.2

Clinical evaluation—Physical examinations including neurologic scoring were performed daily until 14 days after infection and then again at 21 days after infection. Rectal temperatures were recorded twice
daily until 7 days after infection and then daily when samples were obtained. A fever was defined as a rectal temperature > 38.5°C. Neurologic assessments, ranging from grade 0 to 5, were performed by equine clinicians according to a commonly used clinical scoring system described by Furr and Reed.22 When unacceptable conditions occurred, such as recumbency or inability to perform normal body functions such as eating, drinking, urination, and defecation, horses were euthanized. At 21 days after infection, all remaining horses were euthanized. Immediately following euthanasia, CSF was collected from the atlantooccipital space. Full necropsies were performed on horses with neurologic signs.

**Blood, nasal swab specimen, and CSF sampling and processing**—Blood samples and nasal swab specimens were obtained on day –1 and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 21 days after infection. Blood was drawn into 10-mL tubes containing sodium heparin and 5-mL plastic EDTA tubes. Peripheral blood mononuclear cells were isolated from the heparinized blood as recommended by the supplier.26 Aliquots were used immediately for virus isolation. Blood anticoagulated with EDTA was frozen at –20°C, and all samples were processed for qPCR analysis. Blood (10-mL tubes) was collected for serum on day –1 and 7, 14, and 21 days after infection. Blood in the serum tubes was allowed to clot and after centrifugation (3,000 × g, 25°C, 10 minutes), serum was collected and frozen at –20°C for later determination of EHV-1 SNT.

Nasal swab specimens were obtained with 3 sterile, polyester-tipped swabs placed in the nostril contacting the nasal mucosa for up to 2 to 3 seconds. One swab was placed in a 10-mL plain evacuated tube, frozen at –20°C, and processed for qPCR analysis. The other 2 swabs were placed directly into polystyrene tubes containing 2-mL of viral transport medium (10% NCS in PBS solution, supplemented with an antibiotic-antimycotic solution [300 U of penicillin/mL, 300 µg of streptomycin/mL, 0.74 µg of amphotericin B/mL, and 68 µg of enrofloxacin/mL]) and were immediately placed on ice and chilled for > 2 hours, after which they were centrifuged at 500 × g for 10 minutes at 4°C and filtered (2-µm filter). Aliquots were used immediately for viral titrations. Finally, CSF samples (10 mL) were collected into EDTA tubes for cytologic examination at the Cornell University Animal Health Diagnostic Center and tubes without antimicrobials were stored at –80°C for later determination of EHV-1 SNT.

**Virus isolation and titration**—Virus isolation and titrations were performed with RK13 monolayers, which were maintained in MEM supplemented with 10% FBS and an antibiotic-antimycotic solution (300 U of penicillin/mL, 300 µg of streptomycin/mL, and 0.74 µg of amphotericin B/mL) in 24-well plates. For PBMC cocultivation, a 50-µL aliquot of PBMCs at a concentration of 1 × 106 cells/mL was applied to an RK13 monolayer-containing well with 450 µL of medium, and 10-fold dilutions were made. Plates were examined daily for the presence of viral CPE and were recorded as positive or negative. From the nasal swab specimens, 500 µL of nasal swab fluid was applied to an RK13 monolayer and 10-fold dilutions were made. Medium was removed after 1 hour and replaced with fresh medium containing 0.75% methylcellulose. Plates were examined daily for CPE, and after 3 days of incubation, wells were fixed with acetone (90%) and stained with crystal violet. Plaques were counted, and viral titers were calculated.

**qPCR assay**—Five hundred microliters of PBS solution was added to each nasal swab specimen after the specimen was inverted. Thereafter, each tube was vortexed for 10 seconds and centrifuged at 16,000 × g for 5 minutes to create a cell pellet. After removing the swab and supernatant, each pellet was resuspended into 400 µL of PBS solution prior to nucleic-acid extraction. The CSF was centrifuged under the same conditions. To minimize contamination, all pipetting steps were performed under a laminar-flow hood. Nucleic acid extraction from whole blood, nasal swab specimens, and CSF was performed with an automated nucleic-acid extraction system according to the manufacturer’s recommendations. For the automated extraction, 200 µL of each sample (whole blood, resuspended nasal cell pellet, and CSF) was used.

To determine the efficiency of nucleic acid extraction, all samples were analyzed for the presence of the equine GAPDH gene as described.24 In addition, all samples were assayed (with a reported real-time PCR assay) for the presence of the UL33 gene (encoding gB).24,25

Final quantitation of EHV-1 DNA load for the UL33 gene was done as follows: absolute numbers of EHV-1 genomes were extrapolated to a standard curve generated with cloned EHV-1 UL33 fragments. The equine GAPDH CT values were obtained in parallel to the EHV-1 PCR assay. These CT values (the CT value represents the fractional cycle number at which the fluorescence passes the fixed threshold) were used to calculate genome equivalents present per PCR reaction and cell number (after extrapolation to a standard curve generated on cloned equine GAPDH fragments, knowing that every diploid eukaryotic cell has 2 copies of the equine GAPDH pseudogene). Thereafter, the results of EHV-1 gDNA for UL33 gene were expressed as copies/106 cells (either peripheral blood leukocytes or nasal cells).

**Serum neutralization assay**—The serum was inactivated at 56°C for 30 minutes. Serial 2-fold dilutions were made on microtiter plates by use of MEM containing 10% FBS and an antibiotic-antimycotic solution (300 U of penicillin/mL and 300 µg of streptomycin/mL). Then, 80 to 100 TCID50 of EHV-1 was added to each well. As a control, virus was titered at 3-fold dilutions. The samples were incubated at 37°C for 1.5 hours. Then, 5 × 103 RK-13 cells were added to the wells and incubated for 3 days at 37°C. The medium was removed, and the wells were fixed with neutral-buffered 10% formalin, stained with crystal violet, and evaluated microscopically for CPE.

**Statistical analysis**—The data were not normally distributed (eg, some variables were ordinal and others were skewed on histograms), so nonparametric testing via the Wilcoxon rank sum test was performed. Statisti-
cal tests were grouped into 3 sets (clinical signs, live virus and viral DNA in nasal secretions, and live virus and viral DNA in PBMCs) for comparison between the siLuc- versus sigB3-siOri2–treated horses. The maximum measurement (for continuous variables; eg, temperature > 38.5°C or maximum PFU for virus isolation, or lowest CT or copy number for qPCR) along with the number of sample times (or days) on which the result was positive were analyzed.

The data (including all horses [sigB3-siOri2– and siLuc-treated horses]) from a previous study and the present study were tested via logistic regression for a significant ($P < 0.05$) difference between the 2 studies in whether the experimentally challenged horses became clinically affected (EHM). Because no significant difference was found between experiments, the data were combined to compare the onset, magnitude, and duration of viremia (via PBMC PCR assay) by use of 3 logistic regressions in horses that did and did not develop EHM. Statistical analyses were performed by use of commercially available software (Wilcoxon rank-sum tests1 and logistic regression5). A value of $P < 0.05$ was considered significant.

**Results**

**Description of the experimental groups**—The group treated with the sigB3-siOri2 siRNAs originally consisted of 8 horses, including 5 mares and 3 geldings, but an 11-year-old gelding was withdrawn from this group because of fractious behavior and safety risks for the handlers. This horse had received the –24-hour and 12-hour sigB3-siOri2 treatment, along with the EHV-1 infection, and did not develop neurologic signs. The remaining 7 horses had a median age of 7 years (range, 4 to 10 years). The siLuc control group (n = 6) included 2 mares and 4 geldings, with a median estimated age of 10 years (range, 3 to 20 years).

**sigB3-siOri2 treatment and clinical findings**—The instillations with either the infectious virus or siRNAs were well tolerated by most horses. Seven horses required sedation for intranasal administration (3 in the siLuc group and 4 in the sigB3-siOri2 group) between 1 and 5 times. Generally, when the horses were febrile and had signs of depression (starting from 1 day after infection), they no longer resisted the nasal application and only 1 horse required sedation beyond that time. One horse developed transient epistaxis caused by the nasal applicator at the 24-hour postinfection siRNA treatment.

All horses developed serous nasal discharge within 1 to 2 days after infection. One horse in the sigB3-siOri2 group and 2 in the siLuc group developed moderate mucopurulent nasal discharge at 4 to 5 days after infection. All but 3 horses developed mild mandibular lymphadenopathy (2 sigB3-siOri2– and 1 siLuc-treated horses), and moderate mandibular lymph node enlargement was seen in 1 treated horse and 3 control horses.

All horses developed a fever 1 to 2 days after infection (reaching up to 41.6°C) and a second increase in the temperature at 6 days after infection typical of EHV-1 infection (Figure 1). There was no significant difference between groups in the severity of the fever (maximum temperature; $P = 0.25$) and number of time points where the temperature was > 38.5°C ($P = 0.93$).

One horse in the siLuc control group developed ventral abdominal edema 8 days after infection. A thorough physical examination was performed on that day, but no cardiac murmurs or signs of heart failure were noted. A CBC and serum biochemistry panel was within normal limits, and the horse was concluded to have developed edema secondary to vasculitis. This horse subsequently developed ataxia 9 days after infection.

Five horses (2 in the siLuc and 3 in the sigB3-siOri2 group) developed signs of EHM starting 6 to 9 days after infection, with the exception of 1 horse (sigB3-siOri2 group) that had gait deficits (grade...
ataxia) 14 days after infection, which progressed slightly (to grade 1.5) by 21 days after infection. The median age of the horses that developed ataxia was 8 years (range, 5 to 16 years), and that of those without ataxia was 4 years (range, 3 to 20 years). There was no significant \( P = 0.88 \) difference in the severity of the neurologic scores between the siLuc group (median, 0 [range, 0 to 4]) and sigB3-siOri2 group (median, 0 [range, 0 to 4]). The most severely affected horse was found in the sigB3-siOri2 group; that horse developed grade 3.5 ataxia in all 4 limbs on postinfection day 8, which then progressed to grade 4 ataxia. Mild paraparphimosis and slight urine dribbling were noted, but tail and anal tone remained normal. This horse was euthanized on postinfection day 14 due to the severity of disease. One of the horses in the siLuc group also had severe signs and started with a grade 2 ataxia on postinfection day 9 that progressed to grade 4 by postinfection day 11 but had improvement in clinical signs on postinfection day 21 (grade 3). The 2 horses with ataxia scores of 4 received flunixin meglumine (1 mg/kg, q 12 to 24 h) as supportive treatment. One horse (siLuc group) developed mild neurologic signs (grade 2 ataxia on postinfection day 7), which improved to a grade 1 ataxia (postinfection 9), but mild deficits could still be seen at the end of the study. In the sigB3-siOri2 group, 1 horse developed grade 1 ataxia on postinfection day 6, which progressed to grade 3 by postinfection day 9, and then improved on postinfection day 14. The nucleated cell count (reference limit, < 6 cells/µL) and protein concentrations (reference limit, < 100 mg/dL) in the CSF of all horses were within reference limits at the conclusion of the study. However, granular and reactive lymphocytes as well as activated macrophages (vacuolated or containing red-pink cytoplasmic granules) were often detected in the CSF of horses without neurologic disease (granular lymphocytes; median, 3% of the total nucleated cell count [range, 0% to 27%]) and horses with neurologic disease (granular lymphocytes; median, 2% of the total nucleated cell count [range, 0% to 18%]). This provided evidence that antigenic stimulation, consistent with viral infection, occurred in the CNS even in horses without neurologic disease and could be seen in the CSF at postinfection day 21 to 23. Two horses with neurologic disease were positive for EHV-1 by PCR assay \( (1 \times 10^4 \text{ EHV-1 genome copies/mL and } 1 \times 10^6 \text{ EHV-1 genome copies/mL}) \) in the CSF and had an ataxia score \( \geq 3 \) at the time of euthanasia and CSF sampling. The CSF from 1 horse that did not have detectable neurologic signs was also qPCR assay positive \( (2.3 \times 10^3 \text{ EHV-1 genome copies/mL}) \). Histologically, the EHM horses had lymphocytic perivascular cuffs in the CNS (cerebrum, medulla, pons, caudal portion of the medulla, and sometimes the spinal cord). Only 1 horse had evidence of systemic vasculitis.

**sigB3-siOri2 treatment and nasal shedding and viremia**—None of the horses were positive for EHV-1 in the nasal swab specimens or PBMCs (by qPCR assay and VI) prior to infection (day –1). Virus was detected in nasal swab specimens of all horses on postinfection day 1, as determined by co-cultivation on RK13 cells. The nasal swab specimens were generally positive from postinfection days 2 to 5, except for 1 horse that had
an SNT of 2 at the onset of the study (in the sigB3-siOri2 group) and that had extended viral secretion up to postinfection day 9 (Figure 2). There was no significant difference in the maximum amount of virus detected in the nasal swab specimens (P = 0.6) or the number of EHV-1 positive nasal swab specimens (days positive) by virus isolation (P = 0.52) between the sigB3-siOri2- and siLuc (negative control)-treated horses. On the basis of the PBMC cocultivation results, some horses became viremic on postinfection day 3 and all horses had a positive PBMC virus isolation result by postinfection day 6 (Figure 3). Also, there was no significant (P = 0.56) difference in the number of EHV-1-positive PBMC cultures between groups. In addition to virus titrations, a qPCR assay was performed on nasal swab specimens and PBMCs because this is a more sensitive technique to detect viral genome copies. Nasal EHV-1 shedding was detected in most of the horses from postinfection day 1 until the end of the study (postinfection day 21) by qPCR assay (Figure 4), with no significant difference in the maximal nasal EHV-1 DNA copies (P = 0.53) or number of positive swab specimens (P = 0.18) between groups (Table 1). Detection of EHV-1 viremia by qPCR assay revealed EHV-1 genome copies in PBMCs from postinfection day 2 until postinfection day 21 in horses from both groups (Figure 5). No significant differences were observed in the maximum amount of virus detected in the blood (P = 0.36) and number of positive samples (P = 0.56) between groups.

Serum neutralization titers—All horses had a low EHV-1 SNT at the beginning of the study (median for both groups, 16 [range, 2 to 64]), and there was no difference in SNT between groups on postinfection days 7, 14, and 21 (P > 0.21 for all). All horses seroconverted to EHV-1 with an 8- to 512-fold increase in titers at 21 days after infection (Figure 6).

Table 1—Results of VI for EHV-1 and qPCR assay of genome copies of horses after experimental challenge with EHV-1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>siLuc</th>
<th>sigB3-siOri2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swab sample VI (maximum PFUs/mL)</td>
<td>2,200 (12–4,400)</td>
<td>1,540 (96–1,540)</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>3.5 (2–5)</td>
<td>2 (2–9)</td>
</tr>
<tr>
<td>Nasal swab sample qPCR assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum genome copies/10^6 cells</td>
<td>3.9 (\times) 10^7 (1.6 (\times) 10^7–1.0 (\times) 10^8)</td>
<td>5.4 (\times) 10^7 (1.8 (\times) 10^7–5.3 (\times) 10^8)</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>12 (11–13)</td>
<td>12 (12–13)</td>
</tr>
<tr>
<td>PBMC VI (No. of positive samples)</td>
<td>3 (2–5)</td>
<td>4 (2–6)</td>
</tr>
<tr>
<td>PBMC qPCR assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum genome copies/10^6 cells</td>
<td>7,735 (1,510–45,100)</td>
<td>18,700 (606–55,600)</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>7.5 (6–11)</td>
<td>8 (6–10)</td>
</tr>
</tbody>
</table>

Data are median (range).

VI = Virus isolation.

Horses were intranasally administered siLuc (n = 6 horses) or sigB3-siOri2 (7) 24 hours before and 12, 24, 36, and 48 hours after EHV-1 challenge.
Onset, magnitude, and duration of viremia—In the previous study, 5 of 14 EHV-1 infected horses developed EHM, and in the present study, 5 of 13 infected horses developed EHM. Combining the outcome from those 2 studies, in which an identical experimental procedure was used, resulted in 10 of 27 (37%) horses developing EHM. There was no significant difference between the present experiment and the present study as assessed by use of logistic regression and siRNA treatment as an indicator; therefore, we combined the data of both studies to evaluate the magnitude, duration, and onset of viremia in horses that developed EHM versus those that did not. Briefly, the duration (P = 0.3), onset of viremia (P = 0.1), and maximum level (P = 0.8) of viremia, as measured by qPCR assay, were not significantly different between the 10 Ab4-infected horses with EHM and the 17 Ab4-infected horses without neurologic signs (Table 2).

### Discussion

In a previous study conducted by our group, the efficacy of intranasal application of siRNAs against 2 key viral genes of EHV-1 was evaluated. The neuropathogenic strain Ab4 was used to infect horses, and siRNA treatments were given intranasally 12 hours before and 12 hours after infection. In brief, no significant differences were found in EHV-1 nasal secretions or in the level and duration of viremia between horses treated with the siRNAs and control horses. There was, however, a significant difference in the proportions of horses that developed severe EHM and required euthanasia. On the basis of this interesting finding, the purpose of the present study was to repeat the experiment but with a higher dose of siRNAs administered at more frequent intervals than previously tested. Unfortunately, even the administration of a 6.7-fold dose of the anti–EHV-1 siRNA, given 5 times around the time of infection, did not result in any significant differences in clinical signs, nasal secretion, viremia counts, or development of EHM between treated and control horses. At this point, we do not have an explanation for the disappointing results in the present study, compared with our previous study, but potential reasons could include the low numbers of horses per group, the low number of horses that developed EHM, or the method, type, amount, and timing of administration of the siRNAs. Indeed, for the latter, a challenge for the successful application of siRNA will be to determine the administration schedule required for optimal efficacy. Without a true understanding of the kinetics of siRNA stability and the variables that can affect gene silencing, administration of siRNA for clinical use will likely be governed largely by trial and error. In the horses, this approach may be impractical due to the limited experimental group sizes and the amount of material required for in vivo studies. Therefore, an attractive alternative could be to test the efficacy of different doses and delivery schedules first in an in vitro equine nasal mucosa explant study. Such methods have recently been established and validated to study the pathogenesis of EHV-1. In addition, such an explant system could also be used to test the efficacy of siRNAs with or without chemical modifications. Chemical modifications of siRNAs, including lipid-based delivery systems including liposomes, micelles, emulsions, and solid lipid nanoparticles or polymer-based delivery systems, have recently been investigated to overcome the challenges of in vivo siRNA administration. In a previous study, we found that use of lipofectamine for administration of the siRNAs did not have an advantage with regard to protection against EHV-1 disease in mice, compared with use of siRNAs without transfection agent. Therefore, we opted to deliver the siRNAs using only PBS solution in horses of the present study, and this may have contributed to the lack of efficacy.

Because EHV-1 experimental methods in the past have been unreliable at inducing EHM, there is an urgent need for a reliable, repeatable, method to study EHV-1 pathogenesis. In our 2 studies, which we used to combine data on EHM development for logistic regression analyses, intranasal instillation of the neuropathogenic strain Ab4 with a mucosal atomizer was performed in seronegative horses of a variety of ages and breeds. With this method of EHV-1 infection, 37% of infected horses developed neurologic signs. However, we realize that the total number of horses from these 2 studies is inadequate to draw accurate conclusions on the reproducibility of the method and its usefulness to compare treated versus nontreated groups. For example, if this method was used to detect differences between groups in which 20% developed EHM in the treated group versus 30% in the control group, 481 horses would be needed in each group (assuming equal group sizes and α error equals β error of 5%). In the best-case scenario, if 30% of horses in one group had EHM and only 1% had EHM in the other group, a group size of 74 horses would be sufficient. The expense and logistics of horse experiments makes such sample sizes unlikely, and therefore, we feel that randomized blinded field trials are necessary before valid conclusions on treatment or prevention of EHM, including siRNA treatment, can be drawn.

Some work, including field studies as well as experimental studies, has been done revealing that neuropathogenic EHV-1 strains more efficiently induce...
viremia leading to a higher level and longer duration of viremia.30 On the basis of the results, we hypothesized that horses with higher viremia levels for longer periods of time after infection with a neuropathogenic EHV-1 strain are predisposed to develop EHM. However, in the 27 horses experimentally infected with EHV-1 Ab+ by intranasal instillation (the previous study and the present study), a higher level of viremia or a longer duration of viremia prior to the onset of neurologic signs in those horses that developed ataxia and EHM was not detected. Therefore, no evidence was found to support the hypothesis that development of EHM requires a more robust and long-lasting viremia. However, with use of the neuropathogenic virus strain, a certain threshold of viremia may be reached that is more likely to lead to CNS vasculitis. Regardless, the question of why some horses develop EHM when exposed to neuropathogenic EHV-1 strains but others do not is also not fully elucidated. It is likely that host factors, such as age, breed, sex, immunocompetence, and concurrent disease, as well as viral factors are important. For example, young horses infected with a neuropathogenic field strain that did not develop EHM had high EHV-1-specific cytotoxic T-cell counts and lower EHV-1 viral load in the blood after infection.30 This indicates that high EHV-1-specific cytotoxic T-cell counts can be considered protective because they can limit viremia and the development of EHM. In addition, age could have also been an important factor because it has been noted that young horses are less likely to develop EHM whereas aged mares (≥ 20 years) might be more susceptible.11 This might explain the absence of significant differences in viremia levels between horses with and without EHM as was calculated in the present study because horses of various ages were included in these studies.

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e. CAS-1820 X-tractor Gene, Corbett Life Science, Sydney, NSW, Australia.

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