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The effect of siRNA treatment on experimental equine herpesvirus type 1 (EHV-1) infection in horses

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ABSTRACT

Available vaccines fail to induce lasting and protective immunity to equine herpesvirus 1 (EHV-1) associated diseases. RNA interference is a novel approach showing promise for therapeutic use in outbreak situations. This study examined the effect of small interfering RNA (siRNA) on clinical signs as well as the presence of live virus and viral DNA in nasal secretions and peripheral blood mononuclear cells (PBMCs) in horses experimentally infected with EHV-1. siRNA targeting two EHV-1 genes (glycoprotein B and the origin binding protein) was administered 12 h before and 12 h after intranasal infection with EHV-1. Control horses received siRNA targeting firefly luciferase. A significantly smaller proportion (0/10) of horses receiving siRNA targeting viral genes required euthanasia due to intractable neurologic disease as compared to horses in the control group (3/4; $p = 0.01$). There was no significant difference in the presence of live virus or viral DNA in the nasal secretions or PBMCs between the two groups. Future studies are necessary to define the relative contributions of host and virus factors in the development of the neurological form of the infection and to determine an optimal dosing regimen for metaphylactic or therapeutic use of siRNA for treating EHV-1 infection.

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1. Introduction

Equine herpesvirus type 1 (EHV-1) is an alphaherpesvirus that causes usually mild respiratory disease, abortion, and neurologic disease in horses (Harless and Pusterla, 2006). Clinical illness results in an important, albeit poorly quantified, impact on all aspects of the equine industry including reproductive and early neonatal losses, time lost from training and competition and in rare cases, death.

The virus possesses several characteristics that have made the development of an effective vaccine a challenge, including evasion of the host's immune system during the lytic phase of infection (van der Meulen et al., 2006) and establishment of latency in the host when the virus remains dormant and cannot be attacked by humoral or cellular immune effectors (Allen et al., 2008; Slater et al., 1994). Epidemiologic factors also reduce vaccine efficacy; foals are often infected for the first time at a very young age, despite the presence of maternal antibodies (Gilkerson et al., 1998). Conventional vaccines may mitigate clinical signs of respiratory disease and abortion and decrease viral shedding, but have not been found to prevent infection, to stimulate long-lasting immunity or to protect against the neurologic syndrome caused by EHV-1 infection, equine herpes myeloencephalopathy (EHM) (Goodman et al., 2006; Patel et al., 2003). Treatment of horses with clinical disease has traditionally been limited to supportive care. More recently antiviral drugs (including acyclovir and valacyclovir) have been investigated, but poor bioavailability, lack of controlled clinical evidence, and extensive cost of treatment have precluded their wide use in the field (Bentz et al., 2006; Garre et al., 2009, 2007; Henninger et al., 2007; Maxwell et al., 2008).

The failure of conventional drugs and vaccines to produce the desired level of protection has led to the investigation of alternative technologies for prophylaxis and treatment of EHV-1-associated clinical disease. One novel approach that has shown promise both *in vitro* and in other species *in vivo* is the use of RNA interference. RNA interference involves the recruitment of endogenous or exogenous short interfering RNA (siRNA) into RNA-induced silencing complexes (RISCs) resulting in degradation of target mRNA (Stevenson, 2004). For human diseases, *in vivo* studies in Rhesus macaques

showed a beneficial effect of RNA interference in both prevention and treatment of severe acute respiratory syndrome (SARS) coronavirus, including significant decreases in fever, viral load and lung pathology (Li et al., 2005). Moreover, clinical trials in humans were started using siRNA in the treatment of respiratory syncytial virus (RSV) infections (Haasnoot et al., 2007). For equine viruses, *in vitro* studies have shown that siRNA can reduce viral titers in cell cultures infected with equine arteritis virus (Heinrich et al., 2009), African horse sickness virus (Stassen et al., 2007) or Venezuelan equine encephalitis virus (O'Brien, 2007). More specifically for EHV-1, RNA interference has been effective both *in vitro* and *in vivo*, as assessed by a reduction in plaque formation in cell cultures and a reduction in clinical signs (weight loss) and viral replication within lung tissue in a murine model of EHV-1 infection (Fulton et al., 2009).

Evidence from these studies suggests that siRNA might be an ideal agent for protection of in-contact horses during EHV-1 outbreaks, particularly in situations where co-mingling of horses occurs around the time the index case is diagnosed. The objective of this study was to test the hypothesis that metaphylactic intranasal administration of siRNA targeted against critical EHV-1 genes can reduce clinical disease, nasal shedding of virus and viremia in horses experimentally infected with EHV-1.

2. Materials and methods

2.1. Animals and experimental design

The protocol for this study was approved by the Institutional Animal Care and Use Committee at Cornell University. Fourteen horses from at least 6 different breeds were used in this experiment, including 8 geldings and 6 mares ranging in age from 2 to 18 years. One mare approximately 12-years old was determined to be pregnant at the end of the study. The inclusion criteria were an EHV-1 serum neutralizing antibody (SN) titer of ≤1:24 and no spontaneous EHV-1 reactivation during the 6-week observation period prior to the start of the experiment. The horses were kept in two different locations in closed herds with access to pasture and grass hay *ad libitum* prior to the study. Previous vaccination history of the horses was unknown. All horses were moved into a biosecurity level-2 isolation facility, randomly assigned via lottery to treatment (10) and control (4) groups, and allowed to acclimate for 4 days.

This study was conducted in a blinded manner, with all investigators involved in the laboratory assays and clinical evaluations remaining blinded until data was gathered and samples were fully processed at the conclusion of the study. Samples for laboratory assays included blood and nasal swabs obtained on days −1, 1–10, 12, 14 and 21. Physical examinations including neurologic scoring were performed twice a day for the first 48 h post-infection (pi), and on days 6 and 7, and daily on all other days. Physical and neurological examinations were performed by two equine clinicians according to a commonly used clinical scoring system. A fever was defined as a rectal temperature >38.5 °C and neurological scores ranged from grade 0 (no neurological deficits) to grade 5 (recumbency and an inability to rise) (Furr and Reed, 2008).

When unacceptable suffering occurred, such as recumbency or inability to perform normal body functions (eating, drinking, urination and defecation), horses were euthanized immediately and post-mortem examinations performed. At day 21 pi, all remaining horses were euthanized.

2.2. Metaphylactic siRNA treatments and EHV-1 infection

The siRNAs used in this study have been described previously. They target the EHV-1 genes encoding glycoprotein B (sigB3), required for viral entry and cell-to-cell transmission, and the ori-gin binding protein helicase (siOri2), required for replication of the viral genome (Fulton et al., 2009). The control horses received an irrelevant siRNA targeting firefly luciferase (siLuc). The siRNAs were reconstituted according to manufacturer's instructions (Ambion, Austin, TX) and diluted in phosphate-buffered saline (PBS).

Horses were sedated with detomidine (0.01 mg/kg IV) prior to intranasal siRNA treatment and EHV-1 infection. For the treatment, horses received a mixture of siRNAs, consisting of 750 pmol each of sigB3 and siOri2, intranasally using a mucosal atomization device (Wolfe Tory Medical, Salt Lake City, UT). Each dose was administered in a total volume of 10 ml, with 5 ml in each nostril. Metaphylactic administration of siRNA was performed 12 h prior and 12 h after EHV-1 infection (day 0). Control horses received an equal amount of the irrelevant siLuc (1500 pmol) administered in the same way as the treated group. All horses were infected intranasally with 1×10^7 plaque-forming units (PFU) of the EHV-1 strain rAb4 virus (Goodman et al., 2007), using the same application technique as for the siRNA. This strain of EHV-1 possesses the single nucleotide polymorphism (SNP) in the polymerase gene (D752) which is more frequently associated with the development of EHM than strains lacking this mutation (Nugent et al., 2006).

2.3. Sampling and processing

Blood was drawn into serum tubes and tubes containing sodium heparin (Tyco Healthcare Group, Mansfield, MA) on each of the sampling days. Blood in the serum tubes was allowed to clot and after centrifugation ($3000 \times g$, 25 °C, 10 min), serum was collected and frozen at −80 °C for determination of EHV-1 serum neutralization titers. Peripheral blood mononuclear cells (PBMCs) were isolated from 10-ml heparinized blood using Histopaque 1077 (Sigma Aldrich, St. Louis, MO) as recommended by the supplier. Aliquots were used immediately for virus isolation by co-cultivation on RK13 cells, as described below. The remaining PBMCs were frozen at −80 °C for subsequent qPCR processing.

Nasal swabs were obtained using two sterile, polyester-tipped swabs (Puritan Medical Products Company, LLC, Guilford, ME) and placed directly into polystyrene tubes containing 2 ml of viral transport medium: 10% neonatal calf serum in PBS, supplemented with an antibiotic–antimycotic solution (300 U/ml penicillin, 300 μg/ml streptomycin, 0.74 μg/ml amphotericin B and 68 μg/ml enrofloxacin). The nasal swabs were immediately placed on ice and chilled for >2 h, after which they were centrifuged at $500 \times g$ for 10 min at 4 °C. Aliquots were used immediately for viral titrations in RK13 cells, as described below, and the remaining samples were frozen at −80 °C for subsequent qPCR processing.

Finally, cerebrospinal fluid (CSF) samples (10-ml) were collected at the atlanto-occipital space immediately following euthanasia and placed into EDTA tubes for cytology and tubes without anticoagulant. Aliquots were submitted immediately for cytology and the remainder was stored at −80 °C for qPRC. In addition, complete post-mortem examinations were performed on all horses that were euthanized following the development of neurological signs.

2.4. Virus isolation and titration

Virus isolation and titrations were performed using rabbit kidney cell (RK13) monolayers maintained in minimum essential medium (MEM, Mediatech, Herndon, VA) supplemented with 10% FBS (Gemini Bio-products, West Sacramento, CA) and a 1× penicillin, streptomycin, amphotericin B solution (Mediatech, Herndon, VA) in 24-well plates. For the PBMC co-cultivation, a 50 μl aliquot of PBMCs was applied to an RK13 monolayer-containing well with 450 μl medium, and three subsequent 10-fold dilutions were made. Plates were examined daily and evaluated for the presence of viral cytopathic effect (CPE). Plates were recorded as positive or nega-

tive. For the nasal swabs, 500 μl of nasal swab fluid was applied to an RK13 monolayer in 6-well plates, and three subsequent 10-fold dilutions were made. Medium was removed after 1 h and replaced with fresh medium, containing 0.75% methylcellulose (Sigma, St. Louis, MO). Plates were examined daily for CPE and after 3–5 days of incubation, cells were fixed with acetone (90%) and stained with crystal violet. Plaques were counted and viral titers were calculated.

2.5. Quantitative polymerase chain reaction (qPCR)

DNA extraction of PBMCs, nasal swabs, CSF (EZ 96, Omega, Norcross, GA) and tissues (DNeasy 96, Qiagen, Germantown, MD) was done using commercially available kits. qPCR was performed with the Applied Biosystems 7500 FAST real time PCR system (Foster City, CA). The protocol included an initial stage run at 95 °C for 20 s followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Total volume per well was 20 μl, including 10 μl PerfeCTa PCR Fast Mix (Quanta Biosciences, Gaithersburg, MD), 0.18 μl each 50 μM forward and reverse primer, 0.5 μl 10 μM probe, 4.14 μl H_2O and 5 μl of DNA. The target viral gene was EHV-1 IR6 and genome copies in PBMCs were normalized using primers and probes for equine β2-microglobulin (Goodman et al., 2006, 2007). Genome copies in nasal swabs were normalized by adding a standard number of Marek's disease virus (MDV) genome copies (BAC clone pRB-1B) to the lysis buffer and targeting the MDV gene ICP4 (Jarosinski et al., 2002), as previously described (Perkins et al., 2008).

2.6. Statistical analysis

Statistix® 9 and SAS version 9 were used to perform statistical analyses. Data sets were first evaluated for normal distribution using the Kolmogorov–Smirnov test. It was determined that data were not normally distributed, so non-parametric testing including Fisher's exact test and Wilcoxon's rank-sum test was used for subsequent analysis. Statistical tests were grouped into three 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 animals, and one additional set to compare serum neutralization titers and viral load in PBMCs between neurologic versus non-neurologic horses irrespective of treatment group. We then performed a Bonferroni correction to interpretations of the p values on each of these sets.

3. Results

3.1. Description of the experimental groups

The group treated with the sigB3/siOri2 siRNAs included 4 mares and 6 geldings with an average estimated age of 12 years (range 3–18, median 14). The siLuc control group included 2 mares and 2 geldings with an average estimated age of 13 years (range 10–15, median 14). There were no significant differences between the groups at the beginning of the study.

3.2. sigB3/siOri2 treatment resulted in a significantly lower proportion of horses requiring euthanasia for intractable neurologic disease

All horses developed the serous nasal discharge that is characteristic of acute EHV-1 infection within 24–48 h pi. Also, a biphasic increase in temperature was observed, with a first peak on day 1–2 pi (reaching up to 39.5 °C) and a second peak at day 6 pi (Fig. 1). No significant difference was observed in peak temperature ($p = 0.76$) or total number of days of fever ($p = 0.69$) between the two groups. Neurologic signs developed in 3 of 4 siLuc horses and 2 of 10 sigB3/siOri2 horses. Signs of neurologic disease were first
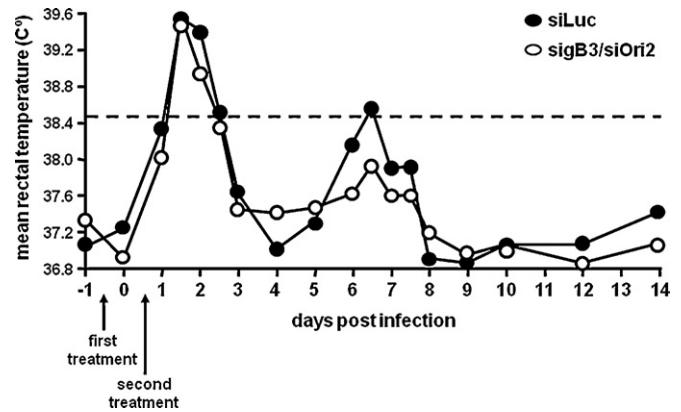

Fig. 1. Mean rectal temperature of horses intranasally administered sigB3/siOri2 or siLuc 12 h before and 12 h after EHV-1 infection (arrows). A fever was considered >38.5 °C and is indicated by the dotted line. Note the biphasic nature of the temperature increases with more modest increase in the second peak; this is typical of neurovirulent EHV-1.

observed on day 7 in one horse in each group (Fig. 2). All siLuc horses that became neurologic met the criteria for euthanasia (2 became recumbent, one was unable to urinate), while none of the sigB3/siOri2 horses did. The proportions of horses developing neurologic signs were not significantly different between the sigB3/siOri2 and siLuc groups ($p = 0.10$). Horses in the siLuc control group had higher median neurological scores ($p = 0.022$), but this did not retain significance when a Bonferroni correction was applied. The difference in the proportion of horses in each group meeting the criteria for euthanasia was significant ($p = 0.01$), and retained significance with Bonferroni correction.

The data on CSF cytology and detection of viral genome copies (by qPCR) in the CSF and spinal cords of the horses that developed neurological signs are summarized in Table 1. The 2 horses in the sigB3/siOri2-treated group that had grade 1 ataxia at the conclusion of the study had no detectable gross or histopathological cen-
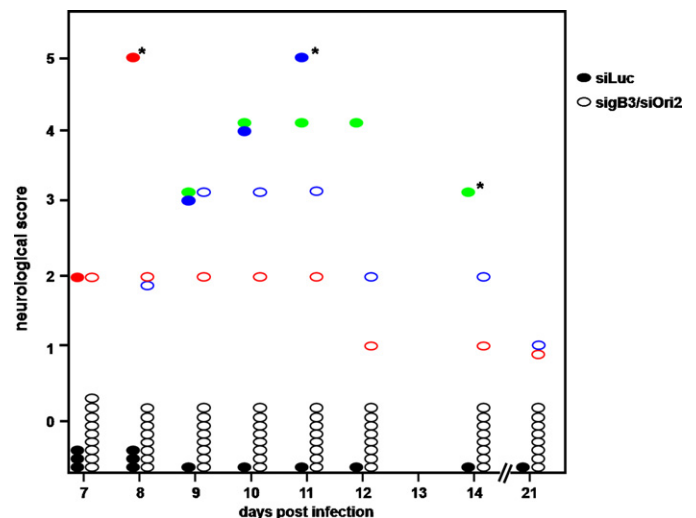

Fig. 2. Neurological scores from day 7 to day 21 post-EHV-1 infection. Neurologic signs were not noted in any horse until day 7 pi. Each symbol represents 1 horse; solid shapes being siLuc-treated horses ($n = 4$: 3 out of 4 developed neurologic signs) and open shapes being sigB3/siOr2-treated horses ($n = 10$: 2 out of 8 developed neurologic signs). Horses that developed neurologic signs were given a color other than black and asterisks indicate the day when a horse was humanely euthanized due to severity of EHM. There was a significant difference between the proportions of horses developing neurologic signs requiring euthanasia in the sigB3/siOri2-treated and control groups ($p = 0.01$). (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

Table 1
Neurological scores, cerebrospinal fluid (CSF) cytology and qPCR.

Horse ID	Group	Neurological score	Day pi of CSF sample	Nucleated cells/µl CSF	CSF total protein (mg/ml)	RBCs/µl CSF	Viral genome copies/ml CSF	Viral genome copies/g spinal cord
Normal reference	–	0	–	<6	<100	<5	0	–
992	siLuc	5	8	2	172	0	1.2×10^5	2.9×10^6
208	siLuc	5	11	2	134	1	1.6×10^5	1.2×10^6
951	siLuc	3	14	6	75	2	0.1×10^5	3.0×10^6
210	sigB3/ori2	1	22	3	66	33	7.3×10^3	0.7×10^6
816	sigB3/ori2	1	24	0	64	0	0	1.6×10^6

tral nervous system abnormalities. A multifocal mild-to-moderate lymphocytic encephalomyelitis and vasculitis consistent with EHM was noted on post-mortem examination of the 3 horses that were euthanized due to severe neurological symptoms. Immunohistochemistry detected abundant EHV-1 antigen within individual endothelial cells in the areas of vasculitis in 1 of the 3 horses tested (data not shown).

3.3. sigB3/siOri2 treatment did not reduce live virus or viral DNA in nasal secretions or PBMCs

Horses in both groups secreted virus only between days 1 and 4 pi (as detected by titration on RK13 cells; Fig. 3A). With qPCR, the presence of EHV-1 DNA in nasal secretions was detected in horses from both groups from day 1 pi to day 14 pi. (Fig. 4A). There was no significant difference in peak virus isolation (median PFU/ml) or days with positive virus isolation from nasal secretions between the two groups. The peak number of EHV-1 genome copies/ml was significantly higher in the siLuc group ($p = 0.024$), although this did not retain significance with Bonferroni correction.

Based on the PBMC co-cultivation results, several horses in both groups were viremic at day 4 pi, and by day 6 pi all horses became positive (Fig. 3B). Quantitative PCR showed the presence of EHV-1 genome copies in PBMCs from day 4 pi to day 9 pi in horses from both grou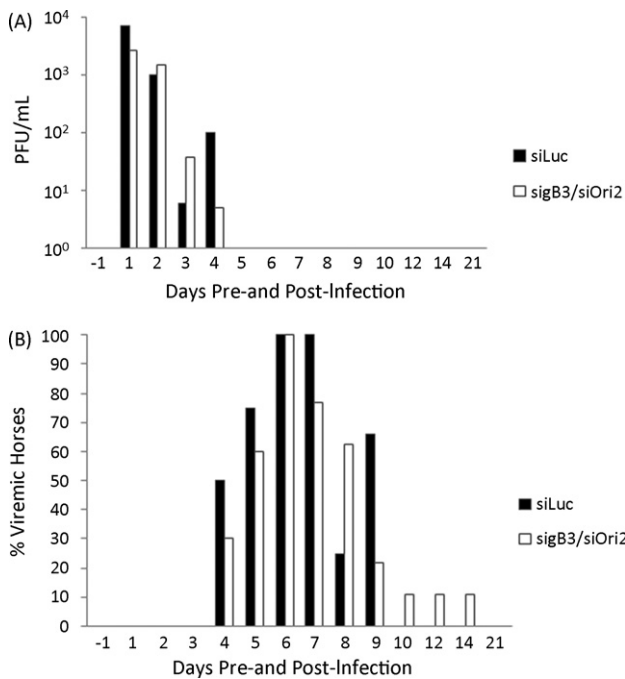ps (Fig. 4B). There was no significant difference in the peak number of EHV-1 genome copies/β2-microglobulin ($p = 0.30$) or number of qPCR positive days between the two groups ($p = 0.29$).

3.4. Serum neutralization (SN) titers and qPCR positive lymphocytes in horses with or without neurological disease

The peak SN titers ($p = 0.052$) and peak EHV-1 genome copies/β2-microglobulin ($p = 0.19$) between neurological and non-neurological horses, irrespective of treatment group, showed no significant difference.

4. Discussion

RNA interference has been studied extensively as a research tool and more recently for its therapeutic potential in treating both cancer and viral diseases. Numerous studies have shown its efficacy in mitigating the effects of viral infection, both *in vitro* and *in vivo*. To the authors' knowledge, this study represents the first attempt to
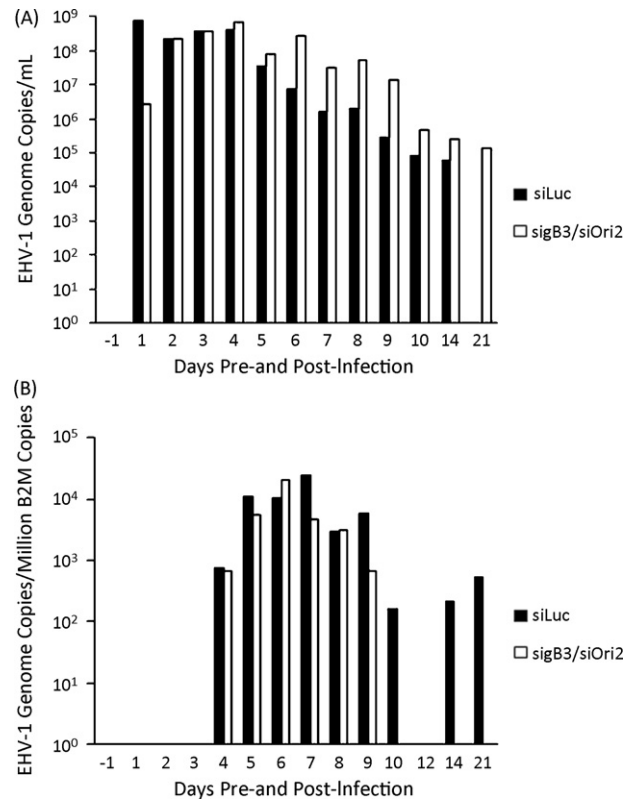

Fig. 3. EHV-1 in equine nasal swabs and PBMCs by virus isolation. (A) Median virus isolation from nasal swabs ($p = 0.76$). Solid bar = siLuc control ($n = 4$) and open bar = sigB3/siOri2 ($n = 10$). (B) Percent of horses positive for virus isolation from PBMCs. Solid bar = siLuc control ($n = 4$) and open bar = sigB3/siOri2 ($n = 10$).

Fig. 4. EHV-1 in equine nasal swabs and PBMCs by qPCR. (A) qPCR on nasal swabs expressed as median EHV-1 genome copies per milliliter of nasal swab media. Solid bar = siLuc control ($n = 4$) and open bar = sigB3/siOri2 ($n = 10$). There were no significant differences between the groups after Bonferroni correction. (B) qPCR on PBMCs expressed as median EHV-1 genome copies per million B2M copies. Solid bar = siLuc control ($n = 4$) and open bar = sigB3/siOri2 ($n = 10$). There were no significant differences between the groups. The sole surviving horse in the siLuc group was viremic on days 14 and 21 pi.

employ RNA interference *in vivo* in horses. The experimental model used in this study closely resembled what occurs in an EHV-1 field outbreak situation in several respects. The population contained horses of various ages, breeds, genders, pregnancy status and likely differing histories of vaccination and exposure. The treatment protocol was metaphylactic, as ensues at the time an index case is diagnosed at the onset of an outbreak (Henninger et al., 2007). However, the use of a non-uniform experimental population as well as the intrinsic nature of EHV-1 infection in horses introduces into the study potentially confounding factors that should be summarized briefly.

In both experimental and field studies it has been suggested that a number of horse-related characteristics, most notably age and immune status can influence the nature and severity of disease resulting from EHV-1 infection (Allen, 2008; Henninger et al., 2007). These factors were either not controlled or not known in this study, and might have influenced the results in ways that are not quantifiable. The epidemiological characteristics of EHV-1 infection also introduce the potential for variability. EHV-1 is endemic in equine populations worldwide, infecting foals at very young ages (Foote et al., 2004) and establishing latent infections that can recrudesce in times of stress (Slater et al., 1994). The stress associated with transporting the study horses from a herd setting into an isolation unit might cause recrudescence of latent virus, possibly confounding some of the results. Although we examined the status of the horses to exclude acute recrudescence (by SN titers and clinical observations), reactivation of latent virus or more severe expression of the disease by co-infection(s) cannot formally be excluded. Our random allocation of the horses would have helped to distribute such confounding evenly between the two groups.

As is often the case with *in vivo* large animal studies, a small sample size made statistical analysis challenging. Our small sample size in the siLuc control group was further affected by the fact that three of the four horses in that group required euthanasia prior to the end of the study. In the sigB3/siOri2-treated group, an impact was made by one horse that showed an unexpectedly severe and unusual course of the challenge infection. On day 3 pi, this horse developed nasal discharge of a character and volume different from that observed in all other horses and from what is typical after EHV-1 infection. Mucopurulent, blood-tinged nasal discharge was produced in very large amounts. At that time a nasopharyngeal swab was obtained and cultured for *Streptococcus equi* subspecies *equi*, but was negative. Additionally, nasal swabs and submandibular lymph node samples obtained post-mortem were negative for *S. equi* on PCR and nasal swabs were negative for equine influenza on PCR. Virus isolated from this horse was sequenced and was identical to the strain administered in this study (rAb4). This horse was one of the two sigB3/siOri2 horses that developed neurologic disease, and alone accounted for extended virus isolation from PBMCs on days 10, 12 and 14 in this group. We were unable to document intercurrent disease, viral recrudescence or viral mutation to justify subject exclusion; the impact of this one horse's unusually severe signs was much greater than it would have been in a larger population.

We hypothesized that treatment with siRNAs against important EHV-1 genes (sigB3/siOri2) would lessen clinical signs as well as the presence of live virus and viral DNA in nasal secretions and PBMCs. The development of intractable neurologic disease necessitating euthanasia was significantly reduced in the sigB3/siOri2-treated group, but unexpectedly the presence of live virus in nasal secretions and PBMCs, and the presence of viral DNA in PBMCs, remained unaffected. Peak content of viral DNA in nasal secretions was lower in the sigB3/siOri2 group, and although this did not retain significance with Bonferroni correction, it lends support for the value of further study on the use of siRNA in EHV-1.

Prior studies including both field outbreaks and experimental infections have shown a relationship between the development

of neurologic signs, increased biphasic temperature, and the magnitude and duration of lymphocyte-associated viremia (Allen and Breathnach, 2006; Henninger et al., 2007). Infection with neurovirulent EHV-1 strains results in higher levels of viremia compared to non-neurovirulent strains (Allen and Breathnach, 2006), and a single nucleotide polymorphism (SNP) in the DNA polymerase gene has been identified to account for this increased level of viremia (Goodman et al., 2007; Nugent et al., 2006; Van de Walle et al., 2009). Characteristics of the host's immune system including a slow rise in EHV-1 specific antibody titers and a low concentration of EHV-1 specific cytotoxic T-lymphocytes prior to infection have also been implicated as risk factors for horses developing EHM (Allen, 2008; Allen and Breathnach, 2006; Henninger et al., 2007). Because all horses in this study were inoculated with the same EHV-1 strain, the neurovirulent rAb4, we expected that both temperature and viremia would be decreased in the sigB3/siOri2-treated group. The findings in the present study showed that neurological disease was mitigated in horses with the EHV-1 specific siRNAs compared to the control group, but no significant reduction in viremia or temperature was observed upon sigB3/siOri2 treatment. There was also no difference in viremia or SN titers of neurologic versus non-neurologic horses, irrespective of the treatment the horses received. These data considered together suggest a much more complex pathogenesis of EHM than our current understanding provides.

While our major finding that neurologic disease was significantly decreased in the EHV-1 specific siRNA treated horses is a highly desirable outcome, it must be interpreted with caution. Reproducing these results in experiments in which host factors are more intensively monitored and controlled would provide additional support for the effectiveness of RNA interference as a suitable therapy to combat EHV-1 infections.

The presumed mechanism of action of intranasally applied siRNAs is uptake into the nasal epithelium, inhibition of initial viral replication and halting of disease progression at the port of viral entry. Much remains to be learned about the cellular uptake and intracellular kinetics of siRNA. Factors thought to influence the duration of effect of siRNA include the rate of cell division and degradation of siRNA by endogenous nucleases. In one *in vivo* murine study looking at these factors, siLuc showed activity of up to 4 weeks in non-dividing liver cells but only 10 days in rapidly dividing tumors cells (Bartlett and Davis, 2006). There are currently no data pertaining to the kinetics of our specific siRNAs in equine epithelial cells; as new research is conducted the effectiveness of administration protocols will gradually be improved.

Potential improvements to siRNA therapy to combat respiratory viruses can include the following: chemical modification of the siRNAs to improve *in vivo* stability and potency; testing of various carriers for optimal delivery, absorption and stability; combining the siRNAs with, e.g. decongestants, especially when nasal discharge is present; modification of the dosing regimen including a higher and/or more frequent dosing; or alternative techniques of administration, such as finer aerosolization. Currently, experiments are planned to chemically modify the sigB3/siOri2 siRNAs and these will be evaluated in experimentally EHV-1-infected horses by administering a higher intranasal dose every 12 h for 3–4 days in the near future.

5. Conclusions

In summary, this study presents the results of the first attempt to use RNA interference with the goal of decreasing clinical disease, viral shedding and cell-associated viremia due to EHV-1 infection in horses. Although there was no significant difference in viral shedding and viremia between the treated and control groups, euthanasia necessitated by intractable neurologic signs was sig-

nificantly reduced after application of EHV-1 specific siRNA, which had been shown previously to interfere with virus replication *in vitro* and in a murine model of EHV-1 infection (Fulton et al., 2009). Given the success seen thus far with the use of siRNA in other models of respiratory disease, we believe that modifications of the siRNAs and/or the dosing regimen used in this study would result in improved outcomes with respect to viremia and virus shedding, and a move towards the ultimate goal of an effective siRNA treatment for this devastating disease. Although progress has been made in recent years towards a better understanding of the disease, additional research to elucidate the relative contribution of viral, host and environmental factors is clearly warranted.

Conflicts of interest

None.

Acknowledgments

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