Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses

George P. Allen, PhD†

Objective—To identify risk factors associated with development of clinical neurologic signs in horses exposed to equine herpesvirus-1 (EHV-1).

Animals—36 adult horses.

Procedures—Blood samples collected before and after challenge inoculation with nonneuropathogenic or neuropathogenic EHV-1 were analyzed for leukocyte-associated viremia, serum neutralizing antibody, and EHV-1–specific cytotoxic T-lymphocyte precursors (CTLPs). Associations between variables and neurologic disease and correlations between age category or breed and development of neurologic disease were examined.

Results—9 horses developed CNS signs (ataxia, hind limb paresis or paralysis, bladder atony, or recumbency). Neurologic deficits were correlated with infection by a neuropathogenic strain of EHV-1, age > 20 years, high postexposure viremic load, and low preexposure concentration of CTLPs. No significant correlations were observed between preinfection titers or horse breed and postinfection development of neurologic signs.

Conclusions and Clinical Relevance—Horses with high concentrations of preexisting CTLPs, regardless of age, strain of virus, or titer, were more likely to control the magnitude of postinfection leukocyte-associated viremia and subsequent development of neurologic disease; therefore, CTLPs appear to be a critical requirement for protective immunity against EHV-1–induced myeloencephalopathy. The importance of achieving immunity related to high concentrations of vaccine-induced CTLPs in horses at high risk for exposure to neuropathogenic strains of EHV-1 is indicated. (Am J Vet Res 2008;69:1595–1600)
identify practical targets for the action of future EHV-1 vaccines and antiviral drugs.

Materials and Methods

Study design—Thirty-six female horses were used for these studies, of which 24 (≥ 20 years of age) were randomly assigned to either of 2 experimental groups of 12 horses each (groups A and B). The remaining 12 younger horses (< 15 years of age) comprised experimental group C. All 24 animals in groups A and C were inoculated with a neuropathogenic strain of EHV-1 (T953). The 12 group B horses were inoculated with a nonneuropathogenic strain of EHV-1 (T262). Statistical comparisons of response variables (eg, development of neurologic signs and magnitude of viremia) were made between experimental horse groups that differed in 1 or more predictor variables (eg, challenge strain of EHV-1, age category, breed, preexposure concentration of CTLPs, and SNA titers).

Experimental animals—Horses used in the study included 36 adult mares ranging in age from 4 to 28 years. Twenty-five horses were Thoroughbred broodmares donated to the Maxwell H. Gluck Equine Research Center by local breeding farms, and 11 were mixed-breed mares from the experimental horse herd maintained by the University of Kentucky equine research farm. None of the horses had been vaccinated against EHV-1 during the year preceding the study. For experimental infection with EHV-1, the horses were assigned to 1 of 3 equal groups and kept in half-acre grassy lots at the university’s equine isolation facilities. Daily health inspections, feeding and watering, preventive health activities (eg, fly and parasite control, hoof care, and vaccinations), and handling and restraint for experimental procedures were performed by farm personnel. Antipyretic, anti-inflammatory, and analgesic medications were administered, as prescribed by the approved experimental protocol, by the author. Veterinary services (including bladder catheterization, euthanasia, and antimicrobial administration) were performed, as needed, by experienced equine practitioners from a local veterinary practice. Following completion of the experimental procedures, the 30 survivors of the experimental infection were returned to the resident horse population of the equine research farm. All experimental procedures were reviewed and approved by the Institutional Care and Use Committee of the University of Kentucky (protocols Nos. 2007-0112 and 850A2005).

Virus strains—A live, low-passage, abortigenic strain of EHV-1 (Ky T262) was used for intranasal inoculation of 12 experimental horses. This strain was isolated from an aborted equine fetus during a large-scale outbreak of herpesviral abortion on a commercial Kentucky Thoroughbred breeding farm in 1975. Its ORF30-2254 genotype was determined via DNA sequencing to be G$_{12,354}$. Both strains of EHV-1 were originally isolated in monolayers of an equine dermal fibroblast cell line and propagated at low multiplicity of infection (0.1) by use of the same cell line for production of the virus stocks used for horse inoculation.

Inoculation of horses with EHV-1—Preinoculation blood and nasopharyngeal samples were collected, and baseline rectal temperatures were recorded. To initiate viral infection, each horse was inoculated intranasally with 10$^7$ plaque-forming units of EHV-1 by use of a fenestrated, 30.5-cm rubber catheter. The course of infection was followed via daily monitoring of clinical signs and rectal temperatures. Nasopharyngeal secretions were collected daily for 7 days with 16-inch, flexible, rayon-tipped swabs and processed for virus isolation by inoculation of monolayers of equine dermal cells. Coagulated and heparinized venous blood samples were collected at 2, 4, 6, 8, 10, 12, 14, and 21 days after inoculation and processed for serum and PBMCs, respectively.

The severity of clinical neurologic signs in the EHV-1 inoculated horses was graded by use of a simplification of the scale described by Mayhew. The observed neurologic deficits were categorized and recorded as either grade 1 (decreased tail muscle tone or toe dragging), grade 2 (any other walking gait abnormality or bladder atony), or grade 3 (recumbency).

Preparation of DNA from PBMCs—The PBMCs were purified as reported by Allen and Breathnach by use of heparinized venous blood samples collected from horses inoculated with EHV-1. For each test sample, RNA-free total DNA was isolated from 5 X 10$^5$ PBMCs by use of a commercial kit designed for purification of genomic DNA from blood leukocytes. Purified DNA was dissolved in 200 μL of sterile water and quantitated via measurement of absorbance at 260 nm.

Real-time PCR quantification of EHV-1 DNA—A real-time PCR assay was used for quantitative detection of EHV-1 DNA in PBMCs following experimental inoculation of EHV-1 as described. The assay was performed by use of detection chemistry and a real-time PCR system with amplification primers and fluorescent detection probes (Appendix). For each DNA sample, triplicate real-time PCR reactions were run, each containing 1 μg of PBMC DNA in 25 μL of complete PCR reaction mixture with 900nM primers designed to amplify a 60-bp fragment of the EHV-1 glycoprotein B gene, and 250nM fluorescent probe for real-time detection of the amplified glycoprotein B sequence. As an endogenous control for normalization of sample-to-sample variations in the amount of target DNA added to the reaction wells, 3 replicates of each sample were run with the glycoprotein B primer-probe set replaced with a primer pair and fluorescent probe for detection of the equine β-actin gene sequence. Amplification consisted of an initial denaturation step of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Quantitative comparison and statistical analysis of the results were performed with specific software and the ΔΔC$_t$ relative quantification method for expressing
observed quantitative differences in amounts of EHV-1 DNA. The quality-control variables (specificity, sensitivity, dynamic range, and efficiency of amplification) and characteristics of the calibrator DNA for the real-time PCR assay have been described.

Determination of CTLP frequency and SNA titer—Estimation of the frequency of EHV-1–specific CTLPs in blood was done by use of a limiting dilution microculture system developed by O’Neill et al. Seven 2-fold dilutions of PBMC responder cells were cultured in vitro with autologous, stimulator PBMCs that expressed EHV-1 antigens. Stimulator PBMCs were generated by infection with 10 plaque-forming units of EHV-1 (Army-183 strain)/cell for 90 minutes followed by a 30-minute treatment at 37°C with 25 µg of mitomycin-C/mL to block proliferation of the stimulator cells during culture. Twenty-four 200-µL replicates of each responder cell dilution were cocultivated in 96-well round-bottomed microplates with 50,000 stimulator PBMCs that functioned as antigen-presenting cells and feeder cells for support of single-cell clonal growth of CTLPs. After 10 days of incubation at 37°C in the presence of 10 units of recombinant human interleukin-2, the induction cultures were assayed for cytolytic activity against chromium 51–labeled, EHV-1– (Army-183 strain) infected lymphoblast targets (pokeweed mitogen–stimulated PBMCs), as described by O’Neill et al. The amount of Cr³⁶ released into each culture well was determined after a 4-hour incubation with 10,000 target cells by counting 200 µL of cell-free supernatant from each well in a gamma counter. Spontaneous Cr³⁶ release by target cells was determined in wells without responder cells, and maximal Cr³⁶ release was measured in wells containing 0.05% NP-40 detergent. A culture well was scored as lysis-positive if the supernatant counts per minute of Cr³⁶ exceeded by ≥ 2 times the mean spontaneous Cr³⁶ release. Frequencies of CTLPs in the blood of each horse were calculated by use of the statistical evaluation software program of Strijbosch et al. The frequencies of CTLPs specific for EHV-1 are expressed as CTLPs per million responder PBMCs. Measurement of EHV-1 SNA titers was performed by use of the microneutralization assay as described.

Statistical analysis—Differences observed between experimental groups of horses in 2 categoric variables (eg, age category and occurrence of neurologic disease or virus strain and occurrence of neurologic disease) were tested for significance via analysis of 2 × 2 contingency tables by use of the Fisher exact test of independence. Significance of differences between experimental groups in numeric variables (eg, viremic load or CTLP frequency) was tested by use of 1-way ANOVA. The significance of an observed linear relationship between the numeric values of preexposure CTLP frequency and viremic load and preexposure CTLP frequency for the 24 EHV-1–inoculated horses was determined (Figure 1). The differences between the 2 age groups of mares in their mean magnitude of EHV-1 viremia were significant after inoculation days 4 through 10. Because the appearance of the scatterplot indicated a fairly strong linear relationship between the individual values of 2 variables, preinfection CTLP frequency and postinfection viremic load, a linear regression line was fitted to the data points by use of the least squares computational method.

The linear relationship between the 2 numeric values may be summarized by the equation, y = a + bx, where y (peak viremic load) = 2.97, b (slope) = −0.027, and x is preinfection CTLP frequency. The correlation coefficient was r = −0.79. Simply stated, the regression line relationship can be reliably used to predict the magnitude of viremia following EHV-1 infection of a horse and its subsequent likelihood for development of neurologic signs, given the preinfection frequency of the horse’s circulating virus-specific CTLPs. By use of the statistic r² as a measure of scatter of the observed data points from the regression line, 62% of the value of the postexposure magnitude of EHV-1 viremia in an exposed horse can be accounted for by the value of the variable preexposure frequency of EHV-1–specific CTLPs. The computed statistical probability that no straight-line relationship existed between the numeric values of pre-exposure CTLPs and postexposure viremic load was P < 0.001. Of particular clinical importance was the observation that all 9 horses that developed neurologic signs after inoculation with a neuropathogenic strain of EHV-1 possessed the combination of low CTLP frequency and high...
The association between vaccine-induced viremia and clinical signs due to EHV-1 infection was examined in horses of different age categories. The null hypothesis was that, following inoculation of horses with neuropathogenic EHV-1, the prevalence of neurologic disease would be the same in young (<15 years) and elderly (>20 years) age categories of horses.

To test the hypothesis, 12 horses belonging to each of the 2 age groups were inoculated intra-nasally with a neuropathogenic strain of EHV-1 (T953) and observed twice daily for neurologic signs. Only 1 horse from the <15 years of age category had post-inoculation neurologic signs, consisting of hind-limb incoordination and dribbling of urine (grade 2) that necessitated the placement of an indwelling urinary catheter. The horse made a full recovery after 2 weeks. In contrast, 6 of the 12 EHV-1 inoculated elderly horses developed clinical signs of neurologic disease. Two of the neurologically affected elderly horses were ataxic only or ataxic with urinary bladder atony (grade 2), whereas the remaining 6 developed lateral recumbency (grade 3) and were euthanatized (P = 0.009); therefore, the null hypothesis was rejected, and results indicated there was a significantly greater risk for post-exposure development of EHV-1 neurologic signs in elderly (>20 years) horses than in horses <15 years of age.

The data were also analyzed for a breed effect on the development of postinfection EHV-1 neurologic disease in the 24 horses. The proportion of horses that developed clinical neurologic signs in the 2 breed categories of horses (Thoroughbred and mixed-breed horses) was not significantly (P = 0.41) different.

To explore the biological basis underlying the greater susceptibility of elderly horses to EHV-1 myeloencephalopathy, the magnitude of postinoculation cell-associated viremia that developed in the 2 age categories of horses was measured and compared (Figure 2). The differences between the 2 age groups in mean magnitude of EHV-1 viremia were significant for post-inoculation days 4 through 10. The magnitude of EHV-1 viremia was greater in the elderly category of horses at all times after inoculation, with concentrations ranging from 10 to 200 times that in the younger horses.

Relationship between EHV-1 genotype and post-exposure development of equine neurologic disease—To test the hypothesis that ORF30 G2,254 strains of EHV-1 pose a greater risk than A2,254 strains for causing EHV-1 myeloencephalopathy, 12 elderly (>20 years) horses were inoculated intranasally with the abortigenic T262 strain of EHV-1 (ORF30 A2,254) and 12 other elderly horses were inoculated with the neuropathogenic T953 strain of EHV-1 (ORF30 G2,254). All 24 of the horses possessed low preinoculation concentrations of EHV-1–specific CTLPs (<40/106 PBMCs). Although none of the aged horses exposed to the abortigenic strain (T262) of EHV-1 developed neurologic signs, 8 of the 12 elderly horses exposed to the neuropathogenic strain (T953) of the herpesvirus developed clinical neurologic disease (2 with grade 2 signs and 6 with grade 3 signs).

The virologic basis for the different degrees of neuropathogenicity of the 2 EHV-1 genotypes was explored by comparison of the magnitude of leukocyte-associated viremia in the 2 experimental treatment groups of horses (Figure 3). The differences in the mean magnitude of EHV-1 viremia resulting from inoculation with the 2 genetic strains of EHV-1 were significant for post-inoculation days 4 through 12. At all sampling times following virus inoculation, the circulating viremic load was significantly greater in the group of 12 horses exposed to the ORF30 G2,254 genotype (T953) of EHV-1.
Relationship between preexposure SNA titer to EHV-1 and postexposure development of neurologic disease—For the 24 horses inoculated with the neuropathogenic strain of EHV-1 (T953), quantitative measurements of EHV-1-specific SNA at the time of virus challenge were performed by use of microneutralization assays (Figure 4). The correlation coefficient, $r$, between the 2 plotted variables was 0.31. The probability that no linear relationship existed between preexposure SNA titer and magnitude of postexposure EHV-1 viremia was $P = 0.144$. No significant association was detected between preexposure SNA titers and the magnitude of postinfection EHV-1 viremia. Likewise, no significant correlation between resistance of horses to EHV-1 neurologic disease and preexposure concentrations of SNA against the virus could be detected.

Discussion

Results of the present study indicated a strong association between the ORF30 genotype ($A_{234}$ or $G_{234}$) of the infecting strain of EHV-1 and the subsequent risk for development of neurologic deficits. Although none of 12 horses inoculated with an ORF30 $A_{234}$ EHV-1 strain developed neurologic signs, 8 of 12 age- and sex-matched horses inoculated with an ORF30 $G_{234}$ strain of EHV-1 developed severe neurologic complications, and 6 required euthanasia. Of the potential risk factors evaluated in this study, infection by an ORF30 $G_{234}$ strain of EHV-1 was most strongly associated with the probability of clinical progression of the viral infection to clinical neurologic disease.

The data also established a strong association between clinical progression of EHV-1 infection to neurologic disease and age category (8 times as great in elderly horses [$>20$ years] than in young to middle-aged adult horses [$<15$ years]). Comparison of the postinoculation viremic load of EHV-1–infected leukocytes in the 12 elderly and 12 young horses revealed that the mean peak magnitude of cell-associated viremia was 100 times as great in the elderly horses. Thus, the quantitative load of circulating virus that follows infection by EHV-1 was defined as a major risk factor for postexposure development of EHV-1 CNS disease.

The relatively greater susceptibility of elderly horses for development of neurologic disease provides an experimental neurologic disease technique that should be useful for assessment of vaccine efficacy against EHV-1 myeloencephalopathy.

Although the specific immune mechanisms required for control of EHV-1 neurologic disease are largely unknown, an immunoeffector mechanism for controlling the amount of cell-associated viremia of other herpesviruses is CTL.\textsuperscript{22,23} Results of the present study indicated that preexposure frequency of EHV-1–specific CTLPs was strongly correlated with postinfection magnitude of cell-associated viremia and was significantly correlated with protective immunity against the myeloencephalopathy caused by infection of horses with neuro-pathogenic strains of EHV-1. These findings suggest a role of CTL immune responses of horses in maintaining immunologic control of EHV-1 viremia. Results therefore were similar to those of Kydd et al\textsuperscript{24} that revealed that resistance to EHV-1 abortion in ponies was associated with high frequencies of preexposure EHV-1–specific CTLPs in blood.\textsuperscript{23} Such results support the view that a critical-mass reservoir of circulating memory CTLs, in place at the moment and location of virus exposure and capable of being activated into functional CTLs with specific cytolytic activity against EHV-1, is required for controlling EHV-1 neurologic disease. It follows that to achieve protective efficacy against EHV-1 myeloencephalopathy by vaccination, the vaccines must be able to stimulate the equine immune response toward the production of such cytolytically functional effector CTLs.

Measurements of EHV-1–specific SNA present in the experimental horses at the time of virus inoculation were performed. The concentration of antibodies prior to virus inoculation did not correlate with protection against challenge with neuropathogenic EHV-1. The results also failed to reveal any significant relationship between preexposure SNA titer and the magnitude of postinfection viremia. The intracellular location of EHV-1 during most of its infection cycle within horses may limit the effectiveness of virus neutralizing antibody in controlling cell-associated EHV-1 viremia.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Relationship between preinfection SNA titer against EHV-1 and either peak magnitude of EHV-1 viremia or development of clinical neurologic disease in 24 horses following exposure to neuropathogenic EHV-1. See figure 1 for key.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Relationship between preexposure SNA titer to EHV-1 and postexposure development of neurologic disease.}
\end{figure}

Reference


Appendix
Real-time PCR oligo primers and fluorescent detection probes used in a study of risk factors for development of neurologic disease after experimental exposure to EHV-1 in horses.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5’ to 3’)</th>
<th>Target DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHV1GB-JN1F</td>
<td>CTG CCC CTG GAG GTG TAC</td>
<td>EHV-1 gB</td>
</tr>
<tr>
<td>EHV1GB-JN1R</td>
<td>TG GCG GCC TGT ATT TCG</td>
<td>EHV-1 gB</td>
</tr>
<tr>
<td>EHV1GB-JN1M1</td>
<td>FAM – CAC CGG CCT GCT AGA CT – NFQ (with minor groove binder adduct)</td>
<td>EHV-1 gB</td>
</tr>
<tr>
<td>EOBACTINIS-JN3F</td>
<td>CCC CGA GGC CCT CCT C</td>
<td>Equine β-actin</td>
</tr>
<tr>
<td>EOBACTINIS-JN3R</td>
<td>GA GTT GAA GGT AGT TTG GTG GAT</td>
<td>Equine β-actin</td>
</tr>
<tr>
<td>EOBACTINIS-JN3M1</td>
<td>FAM – CCC TTC TTC GTG GGC A6 – NFQ (with minor groove binder adduct)</td>
<td>Equine β-actin</td>
</tr>
</tbody>
</table>

FAM = 6-Carboxyfluorescein reporter fluoroprobe. NFQ = Nonfluorescent quencher moiety.