A Single-Nucleotide Polymorphism in a Herpesvirus DNA Polymerase Is Sufficient to Cause Lethal Neurological Disease

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Epidemiological studies have shown that a single-nucleotide polymorphism in the equid herpesvirus type 1 DNA polymerase gene is associated with outbreaks of highly lethal neurological disease in horses. Reverse genetics experiments further demonstrated that a G2254A2254 nucleotide mutation introduced in neurovirulent strain Ab4, which resulted in an asparagine for aspartic acid substitution (D752N752), rendered the virus nonneurovirulent in the equine. Here, we report that the nonneurovirulent strain equid herpesvirus type 1 strain NY03 caused lethal neurological disease in horses after mutation of A2254G2254 (N752D752), thereby providing final proof that the D752 allele in the viral DNA polymerase is necessary and sufficient for expression of the lethal neurovirulent phenotype in the natural host. Although virus shedding was comparable between the N752 and D752 variants, infection with the latter was accompanied by efficient establishment of prolonged cell-associated viremia in peripheral blood mononuclear cells and neurological disease in 2 of 6 animals.

Most viruses, notably members of the herpesvirus family, have established close coevolutionary ties with their hosts [1]. Herpesvirus-induced disease can range from lifelong quiescent and subclinical to fulminant and deadly. Many viral and host factors determine the course of the infection, among which alteration of cellular tropism and immune evasion are preeminent. One prime example of a near-commensally virus-host relationship developed over millions of years is infection with equid herpesvirus type 1 (EHV-1), which usually causes relatively mild disease in affected equids. In some circumstances, however, infection can have devastating and life-threatening consequences, including abortion and highly lethal encephalomyelopathy [2]. The latter is caused by neuronal damage that is consequential to endothelial cell infection, immunopathological reactions, and hypoxia. The neuropathological changes induced by EHV-1 are not necessarily irreversible, but they can result in massive hemorrhage and secondary neuronal death, ultimately resulting in severe paralysis, paraplegia, recumbency, and death of the affected animal [3]. It appears that the neuronal form of EHV-1 disease has become more prevalent in the past decade, and the condition is therefore considered to be a new or reemerging pathological condition caused by a herpesvirus [4].

The pathogenesis of neurologic damage caused by EHV-1 is different from that caused by most of the close relatives of EHV-1 within the alphaherpesviruses, most notably herpes simplex virus type 1 (HSV-1), which can cause lethal encephalitis, especially in immunocompromised children. In the case of this pro-
totypical alphaherpesvirus, damage is done directly to neurons [5]. EHV-1 is apparently unable to cause a lytic infection in neurons and is therefore viewed as a quite unusual alphaherpesvirus [6]. EHV-1 is therefore unlike many of its relatives in humans (HSV-1 and varicella zoster virus) and other animal species (e.g., bovid herpesvirus type 1 and suid herpesvirus type 1), in which a clear neurotropism is noted and latency is established in sensory neurons. Although EHV-1 can establish latency in the trigeminal ganglion, the major and biologically most relevant site of latency is the CD8+ cytotoxic T memory cell [6]. A conundrum of EHV-1 infection has been that, although all EHV-1 strains can cause respiratory disease and abortion, only a subset can induce neurological disease. Recently, a single-nucleotide polymorphism (SNP) in the viral DNA polymerase gene (pol) was identified in a minority of EHV-1 strains and was shown to be associated with lethal neurological disease [7]. Further analyses revealed that mutation of pol sequences in the EHV-1 genome, more specifically an SNP from G2254 to A2254 encoding an asparagine instead of an aspartic acid at position 752 of the enzyme (D752N), resulted in reduced virus replication in peripheral blood mononuclear cells (PBMCs), altered tropism for lymphocyte subsets, and absence of neurological disease. In contrast, virus shedding from the nasal mucosa was identical in the 2 virus variants [8].

Here, we studied whether the G2254 (D752) SNP was not only necessary but also sufficient to confer the neurovirulent phenotype to an a priori nonneurovirulent virus. The results obtained clearly show that the presence of the D752 mutation severely increases the neurovirulence of an EHV-1 strain that was capable of causing abortion but caused otherwise mild disease in nonpregnant animals. On the basis of these findings and our earlier findings, we finally concluded that the naturally occurring D752N752 polymorphism in the EHV-1 DNA pol plays a decisive role with respect to the neuropathogenic potential of this herpesvirus.

METHODS

Statement of animal care. The care and use of animals in this study was approved, performed in accordance with, and supervised by the Cornell University Institutional Animal Care and Use Committee, under the guidelines of the United States Animal Welfare Act.

Viruses and cells. Recombinant viruses were reconstituted from infectious bacterial artificial chromosome clone pNY03, which was derived from an abortogenic EHV-1 strain (NY03, isolated in 2003 from an aborted foal from a farm in New York) by cloning mini-F vector sequences into the gene 71 (gp2) locus of the genome [6, 9]. Virus reconstitution was done in NBL6 cells (ATCC) maintained in minimal essential medium (Mediatech) in the presence of 10% fetal bovine serum (Atlanta Biologicals) by cotransfecting bacterial artificial chromosome and p71 plasmid DNA, which resulted in reinsertion of gene 71 sequences and loss of mini-F vector sequences, essentially as described elsewhere [8–10]. A single-point mutation resulting in the A2254 G2254 mutation in pNY03 was engineered by 2-step Red-mediated mutagenesis [11] with use of primers that harbored the single-point mutation, according to established protocol [8]. A revertant virus in which the original pNY03 sequence was restored was also generated. Virus reconstitution of pNY03_N752, the infectious clone harbor the A2254 G2254 (pNY03_D752) or the revertant (pNY03_D752N) mutation, resulted in parental rNY03_N752, rNY03_D752, or rNY03_D752N recombinant viruses.

Aphidicolin susceptibility assay. The rNY03_N752, mutant rNY03_D752, and revertant rNY03_D752N viruses were grown on rabbit kidney (RK13) cells, which were maintained as described for the NBL6 cells. RK13 cells were infected with 1 plaque-forming unit per cell in the absence or presence of different concentrations of the polymerase-targeting drug aphidicolin [8, 12]. At 72 h after infection and treatment, viral titers were determined by serial 10-fold dilutions on RK13 cells.

Animal studies. Nine horses with anti–EHV-1 serum-neutralizing antibody titers <24 were used in the study. Titers were monitored over the course of at least 6 weeks to exclude spontaneous reactivation of latent EHV-1 infection in the study population. Horses were kept on pasture and fed hay and water ad libitum. Four days before infection with parental rNY03_N752 (n = 3) or the rNY03_D752 mutant (n = 6), horses were transferred into isolation units; they were kept there for the duration of the experiment (21 days). After intranasal inoculation with 1.5 × 103 plaque-forming units of virus, horses were monitored daily and evaluated for (neuro) clinical signs; the identity of the treatment was concealed to the investigators. Blood samples were collected daily in heparinized vacutainers (Beckton-Dickinson), and PBMCs were isolated by density gradient centrifugation over Histopaque 1077 (Sigma), according to the manufacturer’s instructions. Virus was isolated by cocultivation on RK13 cells, and a viremia score was given to each individual horse as follows: a score of 0 indicates that no virus was isolated, a score of 1 indicates that 1–10 plaques were observed using 5 × 104 PBMCs, and a score of 4 indicates plaque observation using as few as 5 × 103 PBMCs. No virus could be isolated in any of the samples in the next 10-fold dilution (5 × 102 PBMCs). In addition, nasal swab samples (Fisher) were collected daily in phosphate-buffered sodium supplemented with fetal bovine serum and antibiotics. DNA isolation and quantitative polymerase chain reaction (qPCR) for detection of virus genomes were performed exactly as described elsewhere [8].
RESULTS

In vitro growth and susceptibility to aphidicolin of the D752 variant. The EHV-1 strain NY03, isolated from an aborted foal and identified to harbor the $A_{2254} (N752)$ pol SNP, was mutated to the presumably neurovirulent $G_{2254} (D752)$ genotype and tested first for its behavior in vitro. As reported for earlier pol mutants of strain Ab4, determination of single-step growth kinetics and plaque area measurements of the generated mutant viruses did not reveal any significant differences between the $N_{752}$ and $D_{752}$ variants (data not shown). Therefore, growth properties of the parental (rNY03_N752), mutant (rNY03_D752), and revertant virus (rNY03_D752N), in the presence of increasing concentrations of aphidicolin (a specific inhibitor of herpesvirus Pol), were tested. The results revealed that the mutant rNY03_D752 virus has decreased susceptibility to aphidicolin, compared with the parental rNY03_N752 and revertant rNY03_D752N virus strains (figure 1). Resistance to aphidicolin at a concentration of 300 nmol/L was significantly different between mutant and parental virus ($P = .001$) and between mutant and revertant virus ($P = .007$) (figure 1). These results are in agreement with our earlier studies in which we used EHV-1 pol mutants that were derived from the originally neurovirulent strain Ab4. Testing of the growth properties in equine cells of the $D_{752}$ and $N_{752}$ variants of Ab4 in the presence of aphidicolin and biochemical data on the efficiency of deoxyribonucleotide triphosphate (dNTP) incorporation with use of recombinant DNA Pol $D_{752}$ and $N_{752}$ proteins clearly showed increased resistance of EHV-1 DNA Pol $D_{752}$ to aphidicolin [8].

In vivo replication and pathogenicity of the D752 variant. Subsequent testing in horses was performed using the rNY03_N752 parental virus (3 horses) and the rNY03_D752 mutant virus (6 horses). Infection with the $N_{752}$ parental virus caused very mild upper respiratory disease with transient fever and a complete absence of neurological symptoms (figure 2 and table 1), similar to observations in earlier experiments with the rNY03_N752 virus in 6 horses (data not shown). Infection with the rNY03_D752 mutant virus also caused a transient fever, and no statistically significant differences between both groups—as determined with Duncan’s multiple range test and Student’s $t$ test with Bonferroni adjustment ($P > .05$)—were detected (figure 2). With regard to neurological symptoms, however, the rNY03_D752 mutant virus caused neurological disease in 2 of 6 animals and death in 1 of the animals (table 1). Horse 206 had rapidly progressive neurological symptoms that led to recumbency necessitating humane euthanasia (day 9 after infection); at that time, a cerebrospinal fluid (CSF) specimen was obtained that had a high number of EHV-1 genome copies. CSF samples were obtained on days 14–28 after infection from all other horses, including horse 208 (whose neurologic signs resolved on day 10 after infection), when horses were clinically healthy. No cytologic abnormalities were seen, but it was apparent that some horses infected with the rNY03_D752 mutant virus had viral genome copies in CSF that were detected by qPCR (table 1). This finding was most likely attributable to a mild CNS vasculitis causing leakage of virus-containing PBMCs into the CSF.

Moreover, neurological disease was accompanied by higher levels of viremia in PBMCs from horses infected with the $D_{752}$ variant than in PBMCs from horses infected with the other variant; the $D_{752}$ variant was also detectable for a longer period (figure 3A). The mean differences in viral load between the rNY03_N752 and the rNY03_D752 groups were statistically significant ($P = .001$, by nonparametric Kruskal-Wallis test). In addition, virus was determined to be present in PBMCs from the rNY03_D752 group on days 2–14 after inoculation, as determined by both qPCR (data not shown) and cocultivation...
of PBMCs with the indicator cell line RK13 (figure 3A). In contrast, viremia was only detected on days 4–12 after inoculation in the rNY03_N752-infected group (figure 3A). A statistically significant difference between the 2 treatment groups was identified with respect to both duration (14 and 12 days in the rNY03_D752 and rNY03_N752 groups, respectively) and magnitude of viremia. The findings showing increased replication of rNY03_D752 in peripheral blood, as determined by viremia levels, were corroborated by evidence of increased replication based on neutralizing antibody titers. When serum samples from infected horses were monitored over the course of the study with use of virus neutralization assays, markedly higher levels of virus-neutralizing antibodies were detected in rNY03_D752-infected horses than in rNY03_N752-infected horses (figure 3B). Taken together, viremia and serological data indicated more robust replication of the rNY03_D752, compared with the rNY03_N752 variant, in peripheral blood; this supports our earlier findings with mutants derived from the originally neurovirulent strain Ab4 [8].

Similar to viremia levels, virus shedding from the nasal mucosa was assessed after virus inoculation. Virus titers were determined by conventional plaque assays, and no statistically significant differences between animals infected with either the rNY03_N752 parental virus or the rNY03_D752 mutant virus were detected (data not shown). Because virus isolation from nasal swab samples by cocultivation is frequently hampered by bacterial or fungal contamination, virus shedding from the nasal mucosa was also determined by qPCR. As shown in figure 4, similar levels of viral DNA were readily detected on days 1–9 after virus inoculation in both groups. Viral loads and shedding were determined to be statistically indistinguishable between both groups by Duncan’s multiple range test and Student’s t test with Bonferroni adjustment (P > .05). These results are in agreement with those of our previous study, in which no statistically significant difference in nasal shedding was observed between Ab4 and the N/D752 Pol variants [8].

**DISCUSSION**

The experiments presented here were designed to further investigate the role of a naturally occurring SNP in herpesvirus pol that was found to be significantly associated with devastating and often lethal neurological disease in the natural host [7]. A versatile and efficient method to manipulate large DNA virus genomes that was developed in our laboratory [11] allowed us to investigate the role of the SNP under controlled experimental conditions. Initially, we found that an a priori neurovirulent strain (Ab4 containing the D752 genotype) could be rendered nonvirulent by mutating the SNP to the N752 genotype. The drastic change in neurovirulence was concomitant with reduced resistance to aphidicolin and increased replication in PBMCs, especially T lymphocytes, in vitro and in vivo [8]. In the present study, we performed the “reverse” experiment, namely to transform a recent isolate identified as a member of the nonneurovirulent EHV-1 clad NY03 into a neurovirulent virus. Similar to the results obtained with the triad of Ab4 mutants generated earlier, we were able to show that NY03 harboring the N752 D752 mutation also exhibits a replicative advantage in select compartments and target cells. However, of importance, the NY03-based N752 variant was still endowed with the capacity to efficiently replicate at the port of virus entry and exit, which is again a corroboration of our earlier results. On the basis of the results of the current study and a previous study by some of the authors [8], we conclude that the described herpesvirus

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**Table 1. Neurological signs, cerebrospinal fluid (CSF) cytology, and quantitative polymerase chain reaction (qPCR) results.**

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Group</th>
<th>Neurological signs</th>
<th>No. of days after inoculation of a CSF sample</th>
<th>Nucleated cell count, cells/μL</th>
<th>Total protein level, mg/mL</th>
<th>RBC count, cells/μL</th>
<th>Viral load, genome copies/mL</th>
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<tr>
<td>Reference ...</td>
<td>Absent</td>
<td>...</td>
<td>&lt;6</td>
<td>&lt;100</td>
<td>&lt;5</td>
<td>0</td>
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<tr>
<td>6</td>
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<td>1</td>
<td>19</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
<td>N752</td>
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<td>22</td>
<td>2</td>
<td>52</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>N752</td>
<td>Absent</td>
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<td>1</td>
<td>41</td>
<td>0</td>
<td>0</td>
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<tr>
<td>18</td>
<td>D752</td>
<td>Absent</td>
<td>14</td>
<td>2</td>
<td>59</td>
<td>0</td>
<td>178</td>
</tr>
<tr>
<td>206†</td>
<td>D752</td>
<td>Severe hind limb ataxia to recumbency (days 7–9)</td>
<td>9</td>
<td>1</td>
<td>140</td>
<td>2</td>
<td>630</td>
</tr>
<tr>
<td>208</td>
<td>D752</td>
<td>Moderate hind limb ataxia; recovered (days 8–9)</td>
<td>28</td>
<td>6</td>
<td>43</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>209</td>
<td>D752</td>
<td>Absent</td>
<td>22</td>
<td>3</td>
<td>62</td>
<td>0</td>
<td>0</td>
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<tr>
<td>211</td>
<td>D752</td>
<td>Absent</td>
<td>22</td>
<td>3</td>
<td>24</td>
<td>1473†</td>
<td>Not done</td>
</tr>
<tr>
<td>214</td>
<td>D752</td>
<td>Absent</td>
<td>22</td>
<td>3</td>
<td>80</td>
<td>0</td>
<td>118</td>
</tr>
</tbody>
</table>

* Neuronal tissue samples were collected from horse 206 at day 9 after inoculation, and viral load was determined by qPCR: brain, 7.3 × 10^8 copies/mg; cervical spinal cord, 2.5 × 10^8 copies/mg; thoracic spinal cord, 6.9 × 10^8 copies/mg; lumbar spinal cord, 4.7 × 10^8 copies/mg. Horse 206 was euthanized on day 9 after inoculation.
† Blood contamination occurred during the spinal tap procedure.
Figure 3. More robust replication in peripheral blood from horses experimentally infected with the mutant equid herpesvirus type 1 rNY03_D752. A, Replication of rNY03 and rNY03_D752 in purified peripheral blood mononuclear cells (PBMCs). Virus was isolated from PBMCs by cocultivation on RK13 cells. Values for individual horses are given for each group; the 2 animals in the rNY03_D752 group that developed neurological symptoms are represented in red (fatal neurological disease) and blue (neurological signs with full recovery). B, Serum neutralization (SN) titers, per group, in samples obtained at the indicated time points. *P < .05.

DNA Pol variants, which represent naturally occurring viruses with a SNP at amino acid position 752 of the polyepitope, exhibit different pathogenic potentials in the natural host. Although SNPs in Pol and their influence on neurovirulence were reported earlier, those mutants were selected for in vitro after treatment with Pol-targeting drugs [13, 14]. Still unresolved, but nonetheless of importance and interest, is the issue of whether neurovirulent D752 EHV-1 strains are (1) continually emerging from the N752 variant and will ultimately replace the less robust nonneurovirulent variant, (2) permanently coexisting with nonneurovirulent N752 viruses, (3) on the way to extinction, or (4) the result of independent, spontaneous mutations from the N752 “original” genotype. Recent investigations on the prevalence of EHV-1 Pol genotypes have shown that 11%–18% of EHV-1 isolates circulating in the North American horse population, depending on the sampling method, seem to represent the neurovirulent variant and that there does not seem to be a sustained trend favoring either genotype [15]. On the basis of these findings and epidemiological data indicating distinct continental lineages, we currently favor the latter 2 aforementioned possibilities. Future studies will closely monitor the prevalence and evolution of the 2 EHV-1 genotypes and pathotypes in horses worldwide to possibly predict and avoid disastrous outbreaks of the lethal neurologic form resulting from virus infection.

Still enigmatic, however, remains the fact that a relatively small mutation outside the catalytic domain of the enzyme could have such a drastic impact on the replicative robustness and tropism of the D752 virus, compared with the N752 virus, in PBMCs (figure 3) [8]. The herpesvirus polymerases show 34%–90% sequence identity, and structural studies of the Pol of HSV-1 revealed 6 structural domains: a pre-NH2 domain; an NH2 domain; a 3′-5′-exonuclease domain; and polymerase palm, fingers, and thumb domains [16]. The D/N752 residue of EHV-1 Pol is homologous to position D751 in HSV-1 Pol and is located between the highly conserved regions II (located in the palm subdomain) and VI (belonging to the fingers domain) [17]. Moreover, this EHV-1 D/N752 Pol variant is immediately followed by the conserved motif A, which together with motifs B and C, forms the incoming dNTP-binding cleft within the active site [16–18]. Similar to previous findings indicating that other viruses that low dNTP pools present in PBMCs can influence virus growth by poor availability of substrate for the elongation of the growing DNA chains [19], an intriguing hypothesis is that the D/N752 Pol variants result in different capabilities of dNTP use in PBMCs. Future studies will therefore concentrate on the ability of the D752 and N752 Pol to elongate DNA in the presence of suboptimal concentrations of dNTP substrates to test the hypothesis of a selective

Figure 4. Virus nasal shedding after experimental infection of horses with parental equid herpesvirus type 1 (EHV-1) rNY03_N752 or mutant EHV-1 rNY03_D752 virus. Virus shedding from nasal mucosa was determined by quantitative polymerase chain reaction. Extended lines indicate standard deviations.
advantage for the D392 Pol variant in the face of limited dNTP pools.

References

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