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# Decreased Infectivity of a Neutralization-Resistant Equine Infectious Anemia Virus Variant Can Be Overcome by Efficient Cell-to-Cell Spread▽

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**Two variants of equine infectious anemia virus (EIAV) that differed in sensitivity to broadly neutralizing antibody were tested in direct competition assays. No differences were observed in the growth curves and relative fitness scores of EIAVs of principal neutralizing domain variants of groups 1 ($EIAV_{PND-1}$) and 5 ($EIAV_{PND-5}$), respectively; however, the neutralization-resistant $EIAV_{PND-5}$ variant was less infectious in single-round replication assays. Infectious center assays indicated similar rates of cell-to-cell spread, which was approximately 1,000-fold more efficient than cell-free infectivity. These data indicate that efficient cell-to-cell spread can overcome the decreased infectivity that may accompany immune escape and should be considered in studies assessing the relative levels of fitness among lentivirus variants, including HIV-1.**

Equine infectious anemia virus (EIAV) induces a persistent lifelong infection characterized by recurrent febrile episodes. Eventually, most horses exert immunological control over replicating virus and enter a prolonged period of clinical quiescence associated with the presence of cytotoxic T cells and broadly neutralizing antibody (bNAb). Over time, however, viral genotypes that resist bNAb evolve, resulting in recrudescence of clinical disease (6, 12). Elucidating mechanisms of viral escape from bNAb is important for the design of effective vaccines for EIAV and related lentiviruses, such as HIV-1.

The V3 region of the EIAV surface envelope glycoprotein (SU) is structurally similar to HIV-1 V1/V2 (5), contains two epitopes recognized by neutralizing monoclonal antibodies (1), and is termed the principal neutralizing domain (PND). Genetic variation in the PND is considered to play an important role in immune escape and EIAV persistence. We previously undertook a longitudinal study of variation in the V2-V4 region of EIAV SU in a pony experimentally inoculated with the virulent Wyo2078 strain of EIAV ($EIAV_{Wyo2078}$) (12). The predominant PND variants clustered into 5 groups, designated PND-1 to PND-5. Genotypes representative of each group were used to generate chimeric infectious molecular clones, designated $EIAV_{PND-1}$ through $EIAV_{PND-5}$, that differed only in the V2-V4 region of SU (12). As infection progressed, the chimeric PND virus variants showed increasing resistance to neutralization by autologous and heterologous sera, such that $EIAV_{PND-1}$ was highly sensitive to neutralization by broadly neutralizing sera, whereas $EIAV_{PND-5}$ was neutralization resistant (12, 14). Genetic differences in the PND region included amino acid substitutions, size variation, and changes in the numbers and locations of predicted N-linked glycosylation sites. Similar changes in HIV-1 *env* that mask immune epitopes have been associated with a loss of virus replicative fitness (8–11), suggesting that resistance to bNAb may incur a cost in virus fitness. In the present study, we used growth competition and infectivity assays to determine if EIAV PND variants that differ in sensitivity to neutralization also differ in replicative capacity *in vitro*.

Direct growth competition experiments were used to determine the relative fitness of chimeric $EIAV_{PND-1}$ and $EIAV_{PND-5}$. Equine dermis (ED; ATCC CCL57) cells were inoculated with equivalent numbers of focus-forming units (FFU) of $EIAV_{PND-1}$ and/or $EIAV_{PND-5}$ at a multiplicity of infection (MOI) of 0.001 for each virus. Supernatant and cells were collected every 3 days, and supernatant virus and provirus copy numbers were determined by quantitative PCR (Q-PCR). The $EIAV_{PND-1}$ and $EIAV_{PND-5}$ genotypes were distinguished using PND-specific primers targeted to the V3 region and a fluorescence probe targeted to a conserved region of *env* (Table 1). The specificity, sensitivity, and efficiency of the Q-PCR assay were tested in reaction mixtures containing various ratios of PND-1 and PND-5 plasmid DNA and by comparing PND-specific primers/probes with EIAV Gag-specific primers/probes by using proviral DNA templates. Viral RNA was isolated from 140 μl of supernatant using the Qiagen viral RNA isolation minikit and amplified by reverse transcription-PCR (RT-PCR) using the TaqMan RNA-to-$C_T$ one-step kit (Applied Biosystems). Each PND genotype was amplified in a separate reaction mixture containing a PND-specific primer pair, and an external standard curve was generated from serial dilutions of $EIAV_{19}$ Env plasmid DNA using common primers and a common probe. Total cellular DNA was isolated with the Qiagen blood cell DNA isolation kit, and the proviral copy number was determined using primers and probes described for viral RNA.

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TABLE 1. Primer and probe sequences used in this study

| Target | Primer or probe[a] | Sequence (5′–3′)[b] |
|---|---|---|
| EIAV Env | Common forward | CGG GGT GTA GAC CAT TTC AA |
| | Common reverse | TGA CAA TGA TCA CTA TCT GTG CAA |
| | Common probe | HEX-TTA TGA GAC CAA TAG AAG CAT GCA-3IALK_FQ |
| | PND-1 reverse | TCC GTA CTC TCA TTT ACA GGG T |
| | PND-5 reverse | CAG TAA GGG TCG GAG GAA CCT G |
| EIAV Gag | Gag forward | AGC CAG GAC ATT TAT CTA GTC AAT GTA GAG CAC C |
| | Gag reverse | GTG CTG ACT CTT CTG TTG TAT CGG GAA AGT TTG |
| | Gag probe | 56-FAM-ACG GGA AGC AAG GGG CTC AAG GGA GGC C-3IALK_FQ |
| Luciferase | Firefly luc forward | TAT GAA GAG ATA CGC CCT GGT T |
| | Firefly luc reverse | GCC CAT ATC GTT TCA TAG CTT C |
| | Renilla luc forward | GCA ATA GTT CAC GCT GAA AGT G |
| | Renilla luc reverse | CAA CAT GGT TTC CAC GAA GAA GT |
| Pseudovirus RNA | WHV PRE forward | CTG TTC CTG TTA ATC AAC CTC TGG |
| | WHV PRE reverse | ATA CAA GGA GGA GAA AAC GAA AGC |
| Equine β2M | E-β2M forward | TCT TTC AGC AAG GAC TGG TCT TT |
| | E-β2M reverse | CTA CCA CAC CAT TGG GAG TAA A |
| | E-β2M probe | 56-ROXN-ATC TTC TGG TCC ATA CTG A-3IAbRQSp |

[a] luc, luciferase; E-β2M, equine β2 microglobulin.
[b] HEX, hexachlorofluorescein; 3IALK_FQ, Iowa Black FQ quencher (IDT); 56-FAM, 5,6-carboxyfluorescein; 56-ROXN, carboxy-X-rhodamine; 3IAbRQSp, Iowa Black FQ quencher (IDT).

The ED cell copy number was quantified using equine β2 M-specific primers and an equine β2 M-specific probe (Table 1). Genomic DNA isolated from $1 \times 10^7$ ED cells was serially diluted to generate a standard curve for cell copy number, and results are reported as numbers of proviral copies/$10^4$ cells.

The relative levels of fitness of $EIAV_{PND-1}$ and $EIAV_{PND-5}$ were compared in the presence and absence of a competitor virus (Fig. 1). Supernatant virus production from $EIAV_{PND-1}$-infected cells was remarkably similar to that of $EIAV_{PND-5}$-infected cells, with respect to both the kinetics of virus production and the maximal output (Fig. 1A). Analysis of proviral copy numbers indicated that the viruses spread through the culture at fairly equivalent rates. Further, the presence of a competitor had no effect on either virus production or cell spread. Competition studies were also performed using virus inocula normalized by virus copy number ($6 \times 10^5$ copies/well).

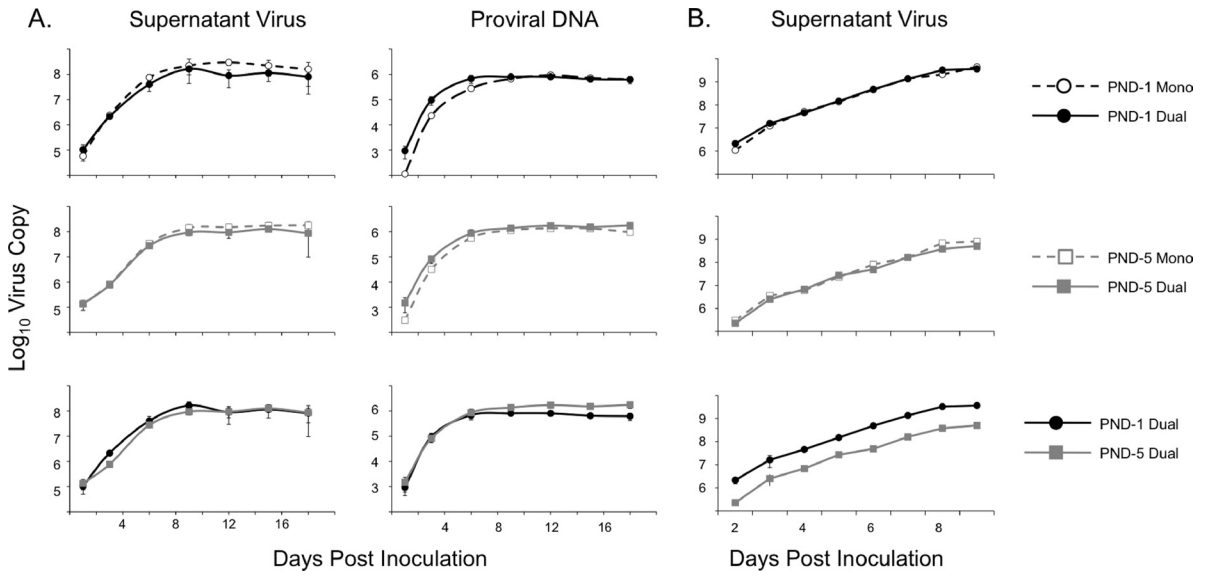


FIG. 1. *In vitro* competition between $EIAV_{PND-1}$ and $EIAV_{PND-5}$. (A) Results of competition studies using inocula normalized by multiplicity of infection. ED cells were inoculated in triplicate with $EIAV_{PND-1}$ and/or $EIAV_{PND-5}$, and the replication kinetics of each virus in singly (Mono) or dually infected cells was assessed by measuring supernatant virion production and provirus-infected cells. (B) Results of competition using inocula normalized by RNA copy number. ED cells were inoculated in triplicate with $EIAV_{PND-1}$ and/or $EIAV_{PND-5}$. Supernatant was collected at sequential times postinfection for quantitation of virion production. Results are the means ± standard deviations of values from triplicate wells assayed in duplicate.

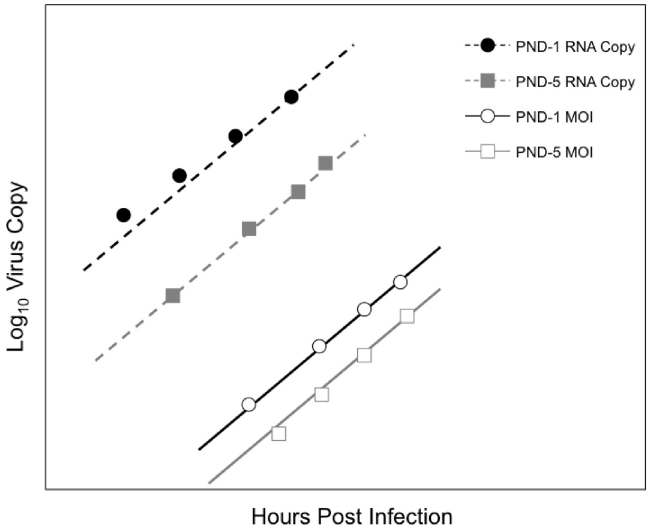


FIG. 2. Fitting of the competition data to the mathematics model. Copy number ($\log_{10}$) of virus as a function of time for the exponential growth phase of the two competition experiments. For each experiment/virus combination, there were four sets of data, results from duplicates of the single infection and those from duplicates of the dual infection. The lines are the estimated models. A fitness difference between EIAV variants PND-1 and PND-5 would manifest as a difference in slopes for the two viruses.

Under these conditions, there was less supernatant virus produced from $EIAV_{PND-5}$-infected cells than from cells infected with $EIAV_{PND-1}$ (Fig. 1B). The decreased virion production appeared to be due to some difference occurring before day 3, since kinetics of virus production were similar after day 3.

To estimate the relative fitness of $EIAV_{PND-1}$ and $EIAV_{PND-5}$, a model of virus exponential growth was fitted to data from all multiround growth assays (Fig. 2). All competition experiments were carried out in duplicate, and each included both single infection with $EIAV_{PND-1}$ or $EIAV_{PND-5}$ and a competition infection with both viruses. There was no obvious difference between the results of the single- and dual-infection experiments, especially during the exponential growth phase, so all data were fit simultaneously to enhance power. Visual examination of the data suggested that the exponential part of the growth curve extended from greater than 24 h to less than 168 h (7 days). During this time, the levels of growth of both viruses were similar, and there was no significant difference between the virus growth rates ($P = 0.51$). Specifically, the fitness of $EIAV_{PND-5}$ relative to that of $EIAV_{PND-1}$ was 0.999, with a 95% confidence interval of 0.995 to 1.003. Therefore, the genetic changes in PND-5 that conferred increased resistance to neutralization were not accompanied by a loss in fitness, as measured in multiple-round growth assays in ED cells.

We next explored possible differences in infectivity between PND-1 and PND-5 genotypes. The $EIAV_{PND-5}$ stocks were consistently less infectious than the $EIAV_{PND-1}$ stocks, as determined by numbers of FFU/ml (Fig. 3A). On a per-milliliter basis, there were, on average, significantly more virus copies ($P = 1 \times 10^{-6}$) but fewer FFU ($P = 2 \times 10^{-9}$) in the $EIAV_{PND-5}$ stocks than in the $EIAV_{PND-1}$ stocks. This resulted in $EIAV_{PND-5}$ having an approximately 3-fold-higher particle/

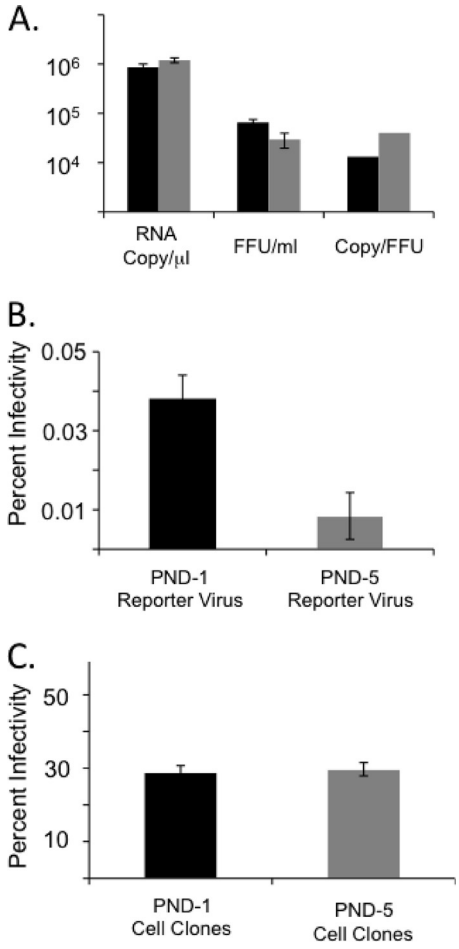


FIG. 3. Infectivity of EIAV variants PND-1 (black bars) and PND-5 (gray bars). (A) Cell-free infectivity of $EIAV_{PND-1}$ and $EIAV_{PND-5}$ stocks. The RNA copy number and infectious titer (focus-forming units/ml) represent the virus effects ± 1 standard deviation, as estimated from a linear mixed-effect model. (B) Single-cycle infectivity of PND-1- and PND-5-pseudotyped viruses carrying the firefly or Renilla luciferase reporter gene. ED cells were coinfected with equal RNA copy numbers of the viruses, and infectivity was calculated as a ratio, the number of infected cells per RNA copy number. (C) Cell-associated infectivity of $EIAV_{PND-1}$ and $EIAV_{PND-5}$, as determined by infectious center assay. Infectivity is calculated as a ratio, the number of infected target cells per donor cell. Two independent cell clones for each virus were assayed in triplicate, and the estimates represent the virus effects ± 1 standard deviation in a linear mixed-effect model, where the cell clone is a random effect.

infectivity ratio than $EIAV_{PND-1}$, suggesting that $EIAV_{PND-5}$ is less infectious than $EIAV_{PND-1}$. To test this hypothesis, we generated replication-defective PND-1 and PND-5 Env-pseudotyped luciferase reporter viruses for analysis in single-cycle infectivity assays. Both firefly luciferase and Renilla luciferase reporter viruses were generated for each PND Env, allowing us to quantify infectivity in coinfected cells by Q-PCR of proviral DNA using luciferase-specific primers. PND-chimeric Env expression plasmids, pLG.PND-1 and pLG.PND-5, were generated by cloning the V2-V4 region of PND-1 or PND-5 into pLGcoSUTM (13), and pseudotyped viruses were produced in HEK 293T cells using an EIAV-based reporter virus system (13). Supernatant pseudovirus copy number was determined by

real-time RT-PCR using the Power SYBR green RNA-to-$C_T$ one-step kit (Applied Biosystems) and primers targeted to the woodchuck hepatitis virus posttranscriptional regulatory element (WHV PRE).

ED cells seeded at $2 \times 10^5$ cells per well in 12-well culture plates were transduced with $2 \times 10^9$ copies of pseudotyped reporter viruses, expressing either the firefly or the Renilla luciferase gene. After 6 h, medium was replaced, and cells were expanded in culture for 3 days. Proviral copy number was quantified by real-time Q-PCR using the Power SYBR green PCR master mix (Applied Biosystems) and primers specific for the firefly or Renilla luciferase gene. The total cell copy number was determined using primers targeted to the equine β2 M gene. Using all experiments, we estimated that 0.038% of PND-1 Env pseudoviruses were infectious and that a significantly smaller 0.0084% of PND-5 Env pseudoviruses were infectious ($P = 0.00013$) (Fig. 3B). This 4.5-fold difference in infectivity is similar to the differences in the particle/infectivity ratios (Fig. 3A) and indicates that changes in the PND region of EIAV SU conferring increased resistance to neutralizing antibody were accompanied by a reduction in virus infectivity.

The PND-5 Env pseudovirus was 4.5-fold less infectious than PND-1, yet the differences in infectivity were not amplified over multiple rounds of virus replication *in vitro*, as indicated by the results of the direct competition assays (Fig. 1). Lentivirus transmission can also occur via cell-to-cell spread, which can be more efficient than cell-free infection (2–4, 7). To determine if cell-to-cell spread compensated for the differences in cell-free infectivity, we used infectious center assays to measure the efficiency of cell-to-cell spread of the PND variants. Cf2th proviral cell clones stably infected with $EIAV_{PND-1}$ or $EIAV_{PND-5}$ (12) were diluted to $1 \times 10^6$ cells per ml, exposed to 25 μg/ml of mitomycin C for 30 min at 37°C, and added to $1 \times 10^5$ indicator ED cells per well in a 12-well culture plate. Replicate wells not seeded with ED target cells were used as controls. Three days after infection, cells were fixed in methanol and foci of EIAV-infected cells were enumerated, following immunohistochemistry using EIAV convalescent-phase immune sera. Infectivity was calculated as the ratio of infected target cell foci to donor cells. Each infectious center assay used two independent cell lines for each virus. The $EIAV_{PND-1}$- and $EIAV_{PND-5}$-infected cells were similar in efficiency of cell-to-cell spread ($P$ value = 0.8), with infectivity of approximately 30% (Fig. 3C). No viable cells were detected in the absence of ED target cells (not shown). We conclude that transmission via cell-to-cell spread is approximately 1,000 times more efficient than transmission via cell-free virus (Fig. 3B and C). This difference in transmission efficiency could easily compensate for differences in cell-free infectivity over multiple rounds of replication. As such, cell-to-cell spread should be considered in studies that examine the relative fitness of lentivirus variants, including HIV-1.

In summary, our results indicate that resistance to neutralization by heterologous antibody was accompanied by a 4-fold loss in cell-free virus infectivity. However, efficient cell-to-cell spread compensated for differences in cell-free infectivity such that PND variants did not differ in relative fitness, as measured in direct competition assays over multiple rounds of replication. Efficient cell-to-cell spread may be an important factor in immune evasion and virus persistence.

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