Disease Induction by Virus Derived from Molecular Clones of Equine Infectious Anemia Virus

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Equine infectious anemia virus (EIAV), a macrophage-tropic lentivirus, causes persistent infections of horses. A number of biologic features, including the rapid development of acute disease, the episodic nature of chronic disease, the propensity for viral genetic variation, and the ability for many infected animals to eventually control virus replication, render EIAV a potentially useful model system for the testing of antiretroviral therapies and vaccine strategies. The utility of the EIAV system has been hampered by the lack of proviral clones that encode promptly pathogenic viral stocks. In this report, we describe the generation and characterization of two infectious molecular clones capable of causing acute clinical syndromes similar to those seen in natural infections. Virus derived from clone p19/wenv17 caused severe debilitating disease at 5 to 7 days postinfection; initial febrile episodes were fatal in two of three infected animals. Virus derived from a second clone, p19/wenv16, caused somewhat milder primary febrile episodes by 10 to 12 days postinfection in two of two infected animals. Virus derived from both clones caused persistent infections such that some animals exhibited chronic equine infectious anemia, characterized by multiple disease episodes. The two virulent clones differ in envelope and rev sequences.

Equine infectious anemia virus (EIAV), a macrophage-tropic lentivirus, causes persistent infections of horses. Following initial exposure to virus, acute disease usually occurs within 1 week to a month, presumably as a result of viral replication in peripheral blood and tissue macrophages (13). The acute phase of the disease is characterized by fever, thrombocytopenia, and high-titer plasma viremia and is usually of relatively short duration (several days), although occasionally some horses develop a particularly severe, fatal form of acute equine anemia (EIA) (9). The chronic or persistent phase of EIA is typified by periodic episodes of clinical illness. These recurrent clinical episodes may be accompanied by anemia, anorexia, and central nervous system depression. EIAV infection does not lead to profound immunologic deficiencies in the host, and virus replication occurs in the presence of an intact immune system. Immunologic studies as well as molecular analyses of viral variants isolated during discrete febrile episodes suggest that chronic EIA is a period of dynamic interaction between host and virus, during which variant viruses, capable of replicating to high titer, occasionally emerge (8, 15, 16). Most recurrent clinical episodes occur within 1 year of the initial infection, and horses then become inapparent carriers. Whole blood taken from healthy carriers efficiently transmits the infection to susceptible horses, and stress and certain immunosuppressive drugs may provoke new rounds of febrile episodes (9). Thus, periods of inapparent infection are an example of viral persistence (and low-level replication) without disease. The immune mechanisms that control virus replication in carrier animals have not been determined. EIAV, with its rapid induction of disease relative to other retroviruses, could be a powerful tool for mutagenesis-based studies of retroviral pathogenesis, particularly as regards the impact of infected monocytes/macrophages. Initial tests of antiviral agents or strategies could be evaluated in a matter of weeks, with the generation of vaccine- or drug-resistant variants followed by monitoring animals for febrile episodes. The ability of many infected horses to gain immunologic control of EIAV replication might also provide important clues for the rational design of retroviral vaccines.

Despite the attractive features of the EIAV model, one serious shortcoming of the system has been the lack of acutely pathogenic molecular clones. While cell culture-adapted, virulent virus stocks have been used in informative studies of antigenic variation, immune selection, and vaccine development, attempts to identify virulent molecular clones from these stocks have, to date, been unsuccessful (17). Field strains of EIAV, such as the highly virulent Wyoming strain, are restricted for replication in cell lines, replicating only in equine macrophages (10, 11, 19), and attempts in our laboratories to generate full-length infectious Wyoming proviruses have been unsuccessful. Therefore, we attempted to generate virulent molecular clones of EIAV by construction of chimeric proviruses containing sequences from an avirulent infectious molecular clone and the Wyoming virus field strain. In this report, we describe two distinct EIAV molecular clones that produce acute disease upon infection of Shetland ponies.

MATERIALS AND METHODS

Construction of chimeric proviruses. As shown in Fig. 1A, the first step in generation of chimeric proviruses was replacement of the 5’ long terminal repeat (LTR) of pSpEia19 with a Wyoming LTR. This was accomplished by amplification of LTR sequences from Wyoming virus-infected equine macrophages, using the primers GCCGCGCGAATTCCTGGGGTTTATGAGGG and CC CCCCTAGATGTAAGGCATCTCGAAGAGAC, corresponding to the 5’ and 3’ sequences of the EIAV LTR and containing HindIII and XbaI cloning sites, respectively. The amplified DNA product was cloned into the HindIII and XbaI sites of the low-copy-number vector pLG338sport (6) to generate pWyoLTR. A full-length provirus was then generated by insertion of the 8.0-kb MluI fragment of pSpEia19 into MluI-digested pWyoLTR. Unique MluI cloning sites are situated in the 5’ region of the EIAV LTR such that the resulting full-length
proviral clone (designated pPW19/wyo5‘ltrmut) contained a 5‘ LTR consisting of Wyoming sequences with a single nucleotide substitution, as shown in Fig. 1B. To generate chimeric clones containing envelope region and 3‘ LTR sequences from Wyoming virus, DNA was amplified from Wyoming virus-infected equine macrophages by using the primers EcoRI1 (CGCGGTCGACGAATTCTGTAGGATCTCGAACAGAC) and 5681 (CCATTTCAAAATTACTTGTTATGAGA) to generate 2.6-kb fragments containing part of the EIAV envelope (env) region and a 3‘ LTR. The amplified envelope region DNA was cloned into the SphI and EcoRI sites of pLG338spor, and individual clones were used to replace the corresponding portion of pPW19/wyo5‘ltrmut to generate a series of chimeric clones as depicted in Fig. 1C. The SphI-to-EcoRI fragments of replication-competent chimeric clones were sequenced by dideoxy sequencing using EIAV-specific oligonucleotide primers.

Generation of virus stocks. D17 cells were cultured in minimal essential medium plus 10% fetal bovine serum (BioWhittaker) in 25-cm² flasks. Cells were maintained at 37°C with 5% CO₂. Eighteen to 24 h prior to transfection, cells were plated at 2.5 × 10⁵ cells per 60-mm-diameter dish. Medium was replaced 2.5 h prior to transfection, which was performed by using the Life Technologies calcium phosphate transfection system. Each plate received 10 μg of plasmid, which was maintained on the cells for 1.5 h at 37°C. Cells were then washed twice with 3 ml of phosphate-buffered saline followed by the addition of fresh medium. Culture supernatants were collected at 24, 48, and 72 h and were monitored for reverse transcriptase (RT) activity (7), using [3H]TTP in place of [32P]TTP. Approximately 1 ml of transfection supernatant was used to infect adherent equine macrophages. Macrophage cultures were established by Ficoll separation of blood cells and plating for 24 h, at which time nonadherent cells were removed by two washes with RPMI plus 10% fresh horse serum (20). Replication-competent virus stocks were identified by development, at 7 to 10 days postinfection, of cytopathic effects accompanied by increases in RT activity.

Pony infections and clinical assays. Shetland ponies were infected intravenously with 0.75 to 1.0 ml of macrophage-derived culture supernatants containing 0.5 × 10⁵ to 1.0 × 10⁶ cpm of RT activity. Rectal temperatures of infected horses were monitored twice a day. Blood samples were obtained pre- and postinfection, and complete blood counts were determined. A single p19wenv/16-derived virus stock was used to infect two Shetland ponies (38056 and 30000). Two different plasmid DNA stocks were prepared for p19/wenv17 and were used to independently derive two virus stocks. One of these was used to infected ponies 38059 and 29987, while the second virus stock was used to infect pony 29990.

RESULTS

Construction and analysis of chimeric proviral clones. We have previously identified several infectious molecular clones of EIAV that while capable of persistent infection of Shetland ponies, are avirulent (17, 20). We also attempted to use similar methods to recover, from equine macrophage cultures, clones representative of the highly virulent Wyoming wild-type field
strain of EIAV. As these attempts were completely unsuccessful, we chose to instead construct chimeric clones in which partial Wyoming wild-type sequences, amplified from infected equine macrophages, were used to replace portions of the avirulent molecular clone, pSPeiav19. As ongoing studies in several laboratories have indicated the potential importance of the EIAV envelope gene and LTR variation in cell tropism and disease (2, 16, 17, 18), we initially targeted these regions in the construction of chimeric proviruses, substituting LTR and env sequences derived from the Wyoming wild-type strain for those of pSPeiav19.

Using the strategy outlined in Fig. 1 and described in Materials and Methods, we inserted a Wyoming wild-type virus LTR sequence (17) into the low-copy-number vector pLG (5). A unique MluI restriction enzyme site, present in essentially all EIAV LTRs, was then used in the construction of a full-length clone containing a Wyoming wild-type-like 5' LTR (with one nucleotide difference from a Wyoming LTR consensus sequence). Concurrently, env and 3' LTR Wyoming wild-type sequences were obtained by DNA amplification techniques and inserted into pLG338 for stable maintenance. Full-length chimeric proviruses were finally assembled by replacing a 2.5-kb SphI-to-EcoRI fragment of pPW19/wyo5'ltmut with the corresponding region (containing most of the env gene and the 3' LTR) with sequences obtained from Wyoming wild-type virus.

Five full-length p19/wyoming chimeras were obtained. These clones were designated the p19/wenv series. LTR and env sequences were determined for each clone. All contained Wyoming wild-type-like 3' LTRs, confirming the rather homogeneous nature of this sequence in the Wyoming virus stock. In contrast, based on sequence analysis of a highly variable region of the surface glycoprotein (SU), two distinct groups of envelope sequences were observed (Fig. 2).

Virus stocks were obtained for each of the five chimeric proviruses by transfection of D17 cells. Low levels of RT activity (100 to 300 cpm/ml) were obtained at 24 to 72 h posttransfection. The presence of RT activity in the supernatants of transfected cells was transient, becoming undetectable after 4 to 5 days. No additional RT activity was observed through 30 days, and no RT activity was noted when culture supernatants were passed to FEA cells, which are highly permissive for pSPeiav19 and other cell culture-adapted EIAV strains. These results were not surprising, as neither D17 cells nor FEA cells are permissive for growth of Wyoming wild-type virus, and suggest that envelope and/or LTR sequences are important determinants of cell tropism. In contrast, virus supernatants from three of the five clones replicated to high titer in adherent cells of the susceptible equine host.
equine monocyte-derived macrophages, as indicated by extensive cytopathic effects and high levels of RT activity. These results suggested that three replication-competent clones were obtained. Two of these clones were then tested for the ability to infect and/or cause disease in Shetland ponies. The two clones chosen for further testing, p19/wenv16 and p19/wenv17, have different SU, transmembrane (TM) and Rev sequences, as shown in Fig. 2.

Infection studies. Macrophage-derived viruses from clones p19/wenv16 and p19/wenv17 were used to infect Shetland ponies. Each pony was infected intravenously with approximately 1 ml of culture supernatant containing $10^5$ RT units of activity. A total of five ponies were infected, and as shown in Fig. 3 and 4; all presented with clinical signs typical of EIAV infection within 1 to 2 weeks. Results of p19/wenv17-derived virus infections were dramatic. Clinical symptoms were severe, with platelet counts declining from a normal level of about $2 \times 10^5$ per mm$^3$ of whole blood to less than $0.5 \times 10^5$ per mm$^3$ of whole blood by 6 to 7 days postinfection. Febrile episodes commenced by 7 to 8 days postinfection, and temperatures remained elevated for up to 10 days. Only one of three p19/wenv17-infected animals recovered from the primary febrile episode; two other animals were euthanized due to the severity of the clinical symptoms. It should be noted that animals 38059 and 29987 were inoculated with independently derived and amplified p19/wenv17 virus stocks. Figure 4 shows the clinical course of disease for two animals infected with clone p19/wenv16-derived virus stocks. The clinical symptoms appeared somewhat later and were milder than noted for p19/wenv17-derived virus. Pony 30000 showed a decreased platelet count by 2 weeks postinfection and experienced two very mild febrile episodes by 3 weeks postinfection. Another series of more severe febrile episodes was noted at 70 to 95 days postinfection. An unusual clinical response occurred in that platelet counts remained low throughout the infection, even during febrile episodes; the platelets also tended to clump severely upon purification. Pony 38056 exhibited one obvious febrile episode at 2 weeks postinfection and then remained afebrile for several months. Each of the five infected animals was antibody positive by 2 weeks postinfection (by agar gel immunodiffusion assay) for EIAV, and viral sequences could be detected in serum by PCR (data not shown).

Sequence analysis. The env region sequences of clones p19/wenv16 and p19/wenv17 were determined and were compared to the sequence of the env region of the avirulent molecular clone, pSPeiav19. As the amino-terminal 89 amino acids of SU (upstream of a unique SphI site used for cloning) are identical, only the region of SU downstream of the SphI site is shown. The most notable differences between the virulent clones p19/wenv16 and p19/wenv17 and the avirulent clone pSPeiav19 occur in two regions of the variable domain of SU. The first region, amino acids 135 to 151, has been previously shown to contain neutralizing epitopes for some EIAV strains (1). The second region, amino acids 250 to 265, was previously identified as a hypervariable region of SU based on sequence analysis of viruses obtained from distinct febrile episodes from an infected animal (16). Other amino acids substitutions are scattered throughout the remainder of SU. Amino acid substitutions also occur in the first hydrophobic transmembrane-spanning domain, the proposed extracellular region of the TM glycoprotein. By in large, conserved amino acid substitutions occur in the TM protein. Another open reading frame in the env region encodes the EIAV Rev protein, which is involved in mRNA splicing and/or transport (12, 14). EIAV Rev is encoded by a multiply spliced mRNA whose first rev coding exon overlaps the amino terminus of env and thus is identical for the clones discussed here.

FIG. 3. Summary of clinical data from three animals infected with viral stocks derived from clone p19/wenv17. Platelet counts are expressed as $10^3$/mm$^3$ of whole blood.

FIG. 4. Summary of clinical data from three animals infected with viral stocks derived from clone p19/wenv16. Platelet counts are expressed as $10^3$/mm$^3$ whole blood.
The second rev coding exon overlaps the TM coding region, and each of the three clones discussed in this report has a unique rev sequence, as shown in Fig. 2B. In contrast to the conserved amino acid substitutions in TM, the majority of amino acid substitutions in rev are nonconservative.

DISCUSSION

In this report, we describe two molecular clones of EIAV that upon transfection of D17 cells produce virus capable of rapidly eliciting febrile episodes in a Shetland pony model. The clones were produced by replacing the LTRs and env region of an avirulent molecular clone with the corresponding sequences obtained from the highly virulent Wyoming wild-type virus. Despite several attempts, we were never able to directly obtain full-length Wyoming virus clones; the reasons for this remain unclear. The reproducibility and rapidity of clinical illness suggests that both of the clones described here (p19/wenv16 and p19/wenv17) produce acutely pathogenic virus and that mutation and in vivo selection events are not necessary for disease induction.

Virus derived from p19/wenv17 induced severe disease in two of three Shetland ponies infected. The onset of febrile disease occurred between 5 and 8 days postinfection, which is typical of infection with a higher titer of Wyoming strain virus. Pony 29990 experienced two febrile episodes with coincident thrombocytopenia, which is typical of either infection with a low titer of Wyoming strain virus or infection with a less virulent strain of EIAV. Virus derived from p19/wenv16 induced a single febrile episode in pony 38056 coincident with thrombocytopenia. Pony 30000 experienced an initial mild febrile episode and a severe thrombocytopenia within the first 2 weeks of infection but, unlike pony 38056, did not experience a rapid recovery of normal platelet levels and instead remained chronically thrombocytopenic. This pony did not experience severe febrile episodes until approximately 11 weeks postinfection. Further studies are needed to determine if this pattern of infection is a characteristic of the p19/wenv16 strain virus or simply individual animal variation in response to the infection. As a result of the method of clone construction, the virulent and avirulent clones described here differ only in env, rev, and TM sequences. In the env region, the most highly divergent sequences are found in SU, in a region previously identified as variable (1, 16). In contrast, amino acid substitutions in TM are nonconservative. The majority of the amino acid substitutions in the rev domain are nonconservative (neutral to polar and/or charged residues) and thus may affect rev biologic activity; a few amino acid substitutions occur in a polar region of EIAV rev previously demonstrated to be required for EIAV rev activity (12).

In summary, the two proviral clones described here may be ideal candidates for mutagenesis-based studies of EIAV pathogenesis, as this pair of clones differ from each other, and from an avirulent molecular clone, in only LTR and env region sequences. In addition, the rapid development of disease by virus derived from these clones should also expedite the testing of vaccine strategies or antiviral therapies directed at EIAV.

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REFERENCES