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Enhanced Sensitivity to Neutralizing Antibodies in a Variant of Equine Infectious Anemia Virus Is Linked to Amino Acid Substitutions in the Surface Unit Envelope Glycoprotein

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Serial passage of the prototype (PR) cell-adapted Wyoming strain of equine infectious anemia virus (EIAV) in fetal donkey dermal (FDD) rather than fetal horse (designated fetal equine kidney [FEK]) cell cultures resulted in the generation of a variant virus strain which produced accelerated cytopathic effects in FDD cells and was 100- to 1,000-fold more sensitive to neutralizing antibodies than its parent. This neutralization-sensitive variant was designated the FDD strain. Although there were differences in glycosylation between the PR and FDD strains, passage of the FDD virus in FEK cells did not reduce its sensitivity to neutralizing antibody. Nucleotide sequencing of the region encoding the surface unit (SU) protein from the FDD strain revealed nine amino acid substitutions compared with the PR strain. Two of these substitutions resulted in changes in the polarity of charge, four caused the introduction of a charged residue, and three had no net change in charge. Nucleotide sequence analysis was extended to the region of the FDD virus genome encoding the extracellular domain of the transmembrane envelope glycoprotein (TM). Unlike the situation with the FDD virus coding region, there were minor variations in nucleotide sequence between individual molecular clones containing this region of the TM gene. Although each clone contained three nucleotide substitutions compared with the PR strain, only one of these was common to all, and this did not affect the amino acid content. Of the remaining two nucleotide substitutions, only one resulted in an amino acid change, and in each case, this change appeared to be conservative. To determine if amino acid substitutions in the SU protein of FDD cell-grown viruses were responsible for the enhanced sensitivity to neutralizing antibodies, chimeric viruses were constructed by using an infectious molecular clone of EIAV. These chimeric viruses contained all of the amino acid substitutions found in the FDD virus strain and were significantly more sensitive to neutralizing antibodies than viruses from the parental (PR) molecular clone. These results demonstrated that sensitivity to neutralizing antibodies in EIAV can be conferred by amino acid residues in the SU protein. However, such amino acid substitutions were not sufficient to enhance cytopathogenicity, as the chimeric viruses did not cause excessive degenerative effects in FDD cells, as was observed with the parental FDD virus strain.

Lentiviruses appear to stimulate weak neutralizing antibody responses in their hosts (1, 27, 31, 32, 41, 55). Only low levels of neutralizing antibodies are usually detected, and in visna virus infections, neutralizing antibodies have been shown to have relatively low binding affinities with their target epitopes (23). These observations suggest that neutralizing epitopes on lentiviruses either are poor immunogens or are somehow resistant to the actions of antibody. An example of a resistance mechanism is seen in caprine arthritis-encephalitis virus, in which binding of neutralizing antibodies is inhibited by sialic acid residues on the surface of the virus particle (16).

Equine infectious anemia virus (EIAV), a lentivirus which causes life-long persistent infections, induces clinical disease characterized by recurrent episodes of fever, edema, anemia, thrombocytopenia, leukopenia, and anorexia (reviewed in references 8 and 20). Determinants on the surface unit (SU) glycoprotein (gp90) of EIAV have been found to be the major targets for antibodies with neutralizing activity, and by the use of monoclonal antibodies (MAbs), three linear neutralizing epitopes have been identified on this glycoprotein (3, 17). Two of these epitopes map to amino acid residues 185 to 194 and 195 to 203, which are in a region of the SU protein of EIAV postulated to be analogous to the principal neutralizing determinant (PND) in gp120 of human immunodeficiency virus type 1 (HIV-1), as it has the potential to form a loop configuration stabilized by disulfide bonds (3, 15, 21, 26, 34). The third linear neutralizing epitope on the EIAV SU protein maps to amino acid residues 264 to 274 (3) and is distinct from the potential loop structure of the PND. Therefore, the EIAV SU protein may contain at least two separate domains capable of inducing the production of neutralizing antibodies.

However, in horses infected with EIAV, the neutralizing domains associated with this virus either are resistant to reactions with antibody or do not promote vigorous humoral responses, as strain-specific neutralizing antibodies are present only in apparently low titers and are usually not detected until 44 to 87 days postinfection (p.i.) (24, 33, 45). In contrast, non-neutralizing antibody responses to the SU protein are routinely detected within 14 to 28 days p.i. (45) and are present in high titers. Although the SU protein constitutes 4% of the virion mass, antibody titers to it are 10 to 100 times greater

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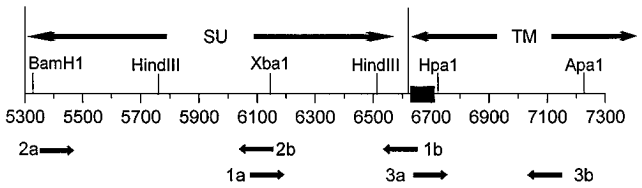

	OLIGONUCLEOTIDE PRIMERS	POSITION 5'	3'
1a	5 'HindIII - TGGGATACATCCAATCAGGC-3'	6087	6106
1b	5' EcoR1 - TAGCGGCTACAATAGCTGC - 3'	6685	6666
2a	5' Nco1 - ATGGTCAGCATCGCATTCTA -3'	5311	5332
2b	5' Kpn1 - CCCAAGAAATATTGTTGGC - 3'	6198	6180
3a	5' GTAGCCGCTACTGCTATTGCTGCT - 3'	6676	6699
3b	5' ACCACTGGTCACTTTCCAGAGGGC -3'	7266	7243

FIG. 1. Oligonucleotide primers used in PCR amplification of SU and TM (extracellular domain) coding sequences from the FDD virus variant of EIAV. Position numbering is according to the complete proviral sequence (GenBank accession number M16575). The black box area represents the fusion domain.

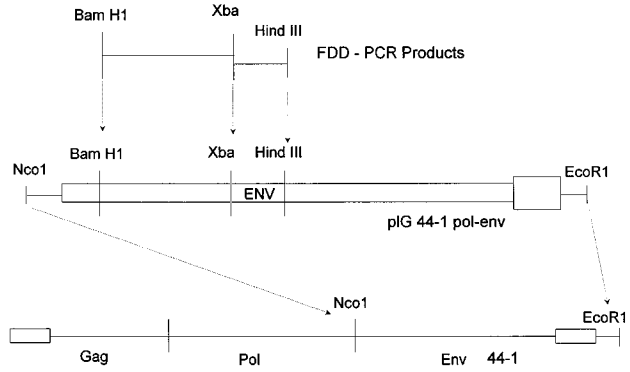

FIG. 2. Construction of a chimeric virus containing FDD virus strain SU protein sequences in the 44-1 infectious molecular clone of EIAV. Primer pairs 2a and 2b and 1a and 1b were used to amplify the region of the FDD virus genome encoding the SU protein. These PCR products were digested with *Bam*HI and *Xba*I or *Xba*I and *Hin*dIII and ligated to form a 1,173-bp fragment, which was inserted into *Bam*HI-*Hin*dIII-digested plG44-1*pol-env* (a vector containing the 3′ region of the *pol* gene, the *env* gene and the 3′ long terminal repeat from infectious molecular clone 44-1) to generate a new plasmid, plG44-1*env*/$FDD_{B\text{-}H}$. In the final manipulation, the *Nco*I-*Eco*RI fragment from plG44-1*env*/$FDD_{B\text{-}H}$ was used to replace an equivalent fragment from 44-1 to create 44-1/$FDD_{B\text{-}H}$.

than those to the core antigen (p26), which constitutes 30% of the virion (33, 35).

A biological variant of the avirulent cell-adapted prototype (PR) Wyoming strain (28) of EIAV has been isolated by serial passage in fetal donkey dermal (FDD) cell cultures (39). This variant, designated the FDD virus strain, differs from the PR strain in terms of cytopathogenicity and sensitivity to neutralizing antibodies. In contrast to the persistent, nonlytic infection observed in fetal equine kidney (FEK) cells, the cell culture-adapted strains of EIAV cause cytopathic effects in FDD cells. These effects are first visible 20 to 22 days p.i. with the PR strain and by 10 to 12 days p.i. with the FDD virus variant (39). In addition to enhanced cytopathic effects, we have recently observed that the FDD virus variant is also 100- to 1,000-fold more sensitive than the PR strain to neutralization by antibody. In this report, the molecular basis of this sensitivity to neutralizing antibody is investigated. A single passage of the FDD virus strain in cells of horse origin did not significantly reduce the titers obtained in neutralization assays. Therefore, the nucleotide sequence of the SU protein and the extracellular domain of the transmembrane protein (TM) were determined, and the role of amino acid substitutions in the SU protein was investigated by construction of chimeric infectious molecular clones of EIAV. These studies demonstrate that sensitivity to neutralizing antibodies in EIAV is conferred by amino acid residues in the SU protein.

MATERIALS AND METHODS

Virus strains and cell cultures. The prototype (PR) cell-adapted Wyoming strain of EIAV (28) was propagated in primary FEK cell cultures as described previously (20). The FDD cell-adapted strain of EIAV was generated by 27 sequential passages of the PR strain in FDD cell cultures (39). FDD cell cultures were maintained as described previously for FEK cells (20).

Preparation of DNA from EIAV-infected cell cultures. FDD virus-infected cell cultures were trypsinized and washed in Hanks' buffered saline solution to remove contaminating proteins from the maintenance medium. The cells were resuspended and lysed in 0.6% sodium dodecyl sulfate (SDS)–50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA. This lysate was digested with RNase (100 μg/ml) at 50°C for 30 min and with proteinase K (0.1 mg/ml) at 50°C for 15 h and then subjected to phenol-chloroform-isoamyl alcohol extractions (25:24:1) to remove protein, followed by two chloroform-isoamyl alcohol (24:1) extractions and ethanol precipitation.

Amplification, cloning, and nucleotide sequencing of FDD virus SU and TM DNA. The SU coding sequences of the FDD virus strain were amplified by PCR from infected-cell DNA with primer pairs 1a and 1b and 2a and 2b (Fig. 1). Approximately 0.9 μg of this DNA was added to a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of the four dioxynucleoside triphosphates, 0.25 μM each of the forward and reverse primers, and 2.5 U of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, Conn.). The final volume of each reaction mixture was adjusted to 100 μl with distilled H_2O. The incubation profile was four cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; and one cycle of 1 min at 94°C, 1 min at 55°C, and 10 min at 72°C.

The PCR product formed with primer pair 2a and 2b was digested with *Nco*I and *Kpn*I to yield an 890-bp fragment. This fragment was cloned into *Nco*I-*Kpn*I-digested pLG338-30 (see below). The 610-bp fragment produced from the reaction with primer pair 1a and 1b was digested with *Xba*I and *Eco*RI, blunt ended with T4 DNA polymerase I, and ligated into either *Sma*I-digested pUC12 or *Hin*cII-digested pUC13. For nucleotide sequencing purposes, the 610-bp fragment produced from the primer pair 1a and 1b reaction was also digested with *Hin*dIII, and the resultant 430-bp *Hin*dIII-*Hin*dIII fragment was cloned into pUC13 which had been digested with *Hin*dIII and treated with calf alkaline phosphatase.

The 585-bp fragment produced from the 3a and 3b primer pair reaction was blunt ended with T4 DNA polymerase I, phosphorylated at the 5′ end with T4 kinase, and ligated into pUC13 which had been digested with *Sma*I and treated with calf alkaline phosphatase. All molecular cloning and enzymatic manipulations were performed by standard protocols (47).

Nucleotide sequences were obtained from double-stranded plasmid DNA by a modified dideoxy technique (5) in conjunction with a series of SU- and TM-specific oligonucleotide primers (44).

Construction of molecular clones and transfection in cell cultures. An outline of the construction of infectious molecular clones of EIAV containing the FDD virus *env* gene sequences is shown in Fig. 2. The generation of the parental infectious molecular clone, designated 44-1, has been described previously (37). Manipulations of EIAV DNA were conducted with a modified version of a plasmid vector designated pLG338 (12). This series of vectors were designed to increase the stability of EIAV proviral sequences in *Escherichia coli* by ameliorating the cytolytic effects resulting from sequences present in the viral *env* gene (12). Modifications to pLG338 were conducted by digesting the plasmid DNA with *Pvu*II and *Hin*dIII and ligating an oligonucleotide linker containing *Nco*I and *Bgl*II restriction endonuclease sites. In order to make substitutions of FDD virus sequences into an infectious molecular clone of EIAV (44-1) more convenient, a 3,449-bp *Nco*I-*Eco*RI fragment encompassing the *pol-env* gene sequences of 44-1 was inserted into pLG338 to create pLG44-1*pol-env*. A *Bam*HI-*Xba*I (823 bp) fragment derived from FDD proviral DNA, amplified by using the 2a and 2b primers (Fig. 1), was ligated to an *Xba*I-*Hin*dIII (350 bp) FDD proviral DNA fragment which had been amplified by using the 1a and 1b primers (Fig. 1). This FDD virus *Bam*HI-*Hin*dIII fragment was then inserted into *Bam*HI-*Hin*dIII-digested pLG44-1*pol-env* to generate pLG44-1*env*/$FDD_{B\text{-}H}$. This construct was then digested with *Nco*I and *Eco*RI, and the resultant 3,449-bp fragment was ligated back into 44-1 to generate 44-1/$FDD_{B\text{-}H}$.

The 44-1 infectious molecular clone (37) selected for these experiments was obtained from proviral DNA isolated from cells infected with a virulent, cell culture-adapted virus stock, designated the pathogenic variant (PV). This PV stock was derived from passage of the avirulent PR strain in ponies, followed by in vitro selection in the presence of neutralizing antiserum to produce an escape

TABLE 1. Growth rates in FEK cells and neutralization characteristics of the PR, PV, and 44-1 strains of EIAV

Strain	Titer ($TCID_{50}$/ml) in FEK cells	Log_{10} neutralization titer[a]	
		PR serum	PV serum
PR	$10^{5.5}$	3.0	3.0
PV	$10^{6.5}$	0.5	4.5
44-1	$10^{5.0}$	2.0	2.0

[a] PR serum was collected from pony 37 at 90 days p.i. PV serum was collected from pony 33 at 90 days p.i. The virus dose was 10^2 $TCID_{50}$.

mutant (19, 45, 46). However, characterization of viruses produced from 44-1 has shown that they closely resemble the PR strain and not the PV strain in terms of neutralization profile (Table 1), growth rates in FEK cell cultures (Table 1), and lack of virulence in ponies (37). Therefore, 44-1 probably represents a PR-like subpopulation present in the PV viral stock. The SU coding region of 44-1 contains four amino acid changes compared with the published PR sequence (37, 44). Substitution of a *Bam*HI-*Hin*dIII fragment from the FDD virus strain would remove three of these changes in 44-1, leaving only a valine to isoleucine substitution at position 420, which, as shown in Fig. 4, is also present in the FDD virus strain. Therefore, hybrid molecular clones between 44-1 and an $FDD/_{B\text{-}H}$ *env* gene fragment would contain all the amino acid substitutions identified in the FDD virus SU protein.

Plasmid DNA preparations from complete molecular clones of EIAV were purified with minor modifications by a polyethylene glycol precipitation procedure described previously (25). These modifications included (i) a phenol-chloroform-isoamyl alcohol (25:24:1) extraction of the supernatant obtained after centrifugation of the bacterial cell lysate and (ii) digestion of RNase A-treated nucleic acids with proteinase K (250 μg/ml) in the presence of 0.25% SDS for 30 min at 37°C. Purified plasmids containing EIAV proviral DNA were introduced into FEK or FDD cell cultures by calcium phosphate-mediated transfection (7).

Antiserum and neutralization assays. MAbs to epitopes A (MAb gp90-A) and E (MAb gp90-E) on the SU protein of EIAV have been described previously (3, 17). Polyclonal antiserum to EIAV was obtained from ponies infected with either the PR strain (ponies 37, 78, and 918) or the PV strain (pony 33). For neutralization assays, serum samples were collected between 90 and 100 days p.i. to ensure the presence of strain-specific neutralization antibodies (45, 47). All serum samples were incubated at 58°C for 30 min to inactivate complement or residual EIAV (4). Neutralization assays were of the β type, in which various dilutions of serum were mixed with a constant dose ($10^{1.5}$ or $10^{2.0}$ 50% tissue culture infectious doses [$TCID_{50}$] per reaction) of virus. In these assays, 0.1 ml of virus and 0.1 ml of each serum dilution were incubated at 37°C before being added to monolayers of FEK cell cultures contained in cell culture tubes (16 by 125 mm; Corning Inc., Corning, N.Y.). The cells were incubated for 1 h prior to the addition of modified Eagle's medium containing 3% fetal bovine serum, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10 mM $NaHCO_3$, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Cell cultures were maintained for 3 to 4 weeks before the culture fluids were assayed for the presence of reverse transcriptase activity (9). Neutralizing antibody titers (log_{10} units) were calculated from the highest serum dilution at which reverse transcriptase activity could not be detected.

Analysis of SU protein of the PR and FDD virus strains before and after glycosidase treatment. The PR and FDD virus strains were propagated in FEK and FDD cells, respectively, and purified by glycerol density gradient centrifugation as described previously (30). Purified virions (40 μg) in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS were boiled for 3 min and adjusted to 0.02 M sodium phosphate buffer (pH 6.0), 0.01 M NaN_3, 0.005 M EDTA, and 1% Triton X-100 before the addition of 1.6 U of *N*-glycosidase F and 1.6 mU of *O*-glycosidase (Boehringer Mannheim, Indianapolis, Ind.). Following a 15-h incubation at 37°C, a further 1.2 U of *N*-glycosidase F and 1.2 mU of *O*-glycosidase were added, and incubation at 37°C was continued for a further 6 h. Glycosylated and deglycosylated purified virus preparations were analyzed by polyacrylamide gel electrophoresis (PAGE) and immunoblotting (45). The SU protein was visualized by using the gp90 E MAb in conjunction with a goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, Mo.) and 3,3′,5,5′-tetramethylbenzidene (Promega, Madison, Wis.) as the substrate.

RESULTS

Analysis by PAGE and immunoblotting revealed minor differences in electrophoretic mobilities between the SU protein from purified preparations of the FDD and PR virus strains (Fig. 3). Aliquots of these purified viral preparations were incubated with *N*-glycosidase F and *O*-glycosidase. Although a

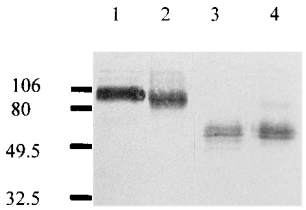

FIG. 3. Immunoblot analysis of the SU protein from purified preparations of the PR and FDD virus strains. Both viral preparations were examined before and after treatment with *N*-glycosidase F and *O*-glycosidase. Following electrophoretic transfer from a polyacrylamide gel to a nitrocellulose filter, the SU protein was detected with MAb gp90-E and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin as the secondary antibody. The SU protein before glycosidase treatment is shown in lanes 1 (PR) and 2 (FDD) and after treatment in lanes 3 (PR) and 4 (FDD). The positions of molecular mass markers (in kilodaltons) are indicated on the left.

proportion of the SU molecule appeared to be resistant to complete deglycosylation, as evidenced by some heterogeneity in the banding pattern in polyacrylamide gels (Fig. 3), the overall effect was that the electrophoretic mobilities of the FDD and PR virus SU proteins became very similar (Fig. 3). This suggested that there was differential glycosylation between the FDD virus strain grown in FDD cells and the PR strain grown in FEK cells (Fig. 3). However, variations in host cell-specified glycosylation were not responsible for the enhanced neutralization characteristics of the FDD virus strain, as passage of this virus in FEK cells did not significantly reduce its reactivity with neutralizing antibody. At a virus dose of $10^{1.5}$ $TCID_{50}$ with serum from pony 918, the log_{10} neutralizing titer was 1.8, 4.5, and 4.2 for virus strains PR, FDD, and FDD that had been passaged once in FEK cells, respectively. These data suggested that the mechanism for sensitivity to neutralizing antibody in the FDD virus strain was probably genetic and not epigenetic in origin.

The viral components with the highest probability of dictating sensitivity to neutralization were considered to be the SU and the transmembrane (TM) proteins. As described above, the SU protein contains the known neutralizing determinants on EIAV (3, 17), while mutations in the TM protein have been observed to affect the sensitivity to neutralization of HIV-1 (2, 40, 56). Consequently, sequences in the FDD virus strain encoding these proteins were selected to be the focus of further studies. Regions of the FDD virus genome encoding the entire SU protein and the extracellular domain of the TM protein were amplified by PCR and cloned into either pLG338 or pUC12 and -13. Nucleotide sequence analysis of molecular clones encompassing the coding region of the SU protein revealed nine amino acid substitutions (Fig. 4) compared with the published sequences for the PR strain (44). Of these substitutions, at least five have been observed to occur in different isolates of the PR strain after serial passage in ponies (36, 37). The amino acid changes were distributed in different domains of the SU protein. A single amino acid substitution (glycine to glutamic acid at position 81), which has not been reported in pony-passaged isolates of the PR strain (36–38) or in the virulent Wyoming strain of EIAV (37), was identified in the conserved N-terminal domain (amino acid positions 1 to 137). Two amino acid substitutions (valine to isoleucine at position 420 and glutamine to glutamic acid at position 394) were observed in the conserved C-terminal domain (amino acid positions 361 to 438), of which the substitution at position 394, similar to the one at position 81, has not been reported previously (36–38). The remaining six amino acid substitutions (threonine to methionine at position 194, glutamic acid to

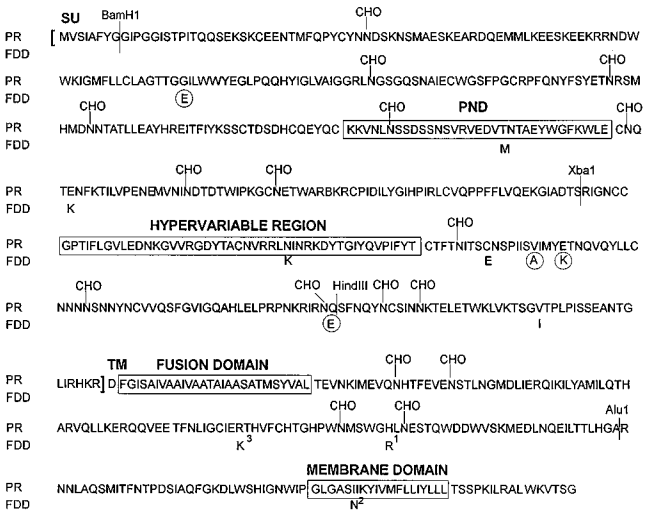

FIG. 4. Amino acid sequence of the SU protein and the extracellular domain of the TM protein of FDD virus. Comparison of the amino acid sequence (single-letter code) of the PR and FDD virus strains of EIAV. For the FDD virus strain, only substitutions are shown. In the case of the SU glycoprotein, the substitutions which are circled have not been observed in pony-passaged isolates of the PR strain or in the virulent Wyoming strain of EIAV. Although each TM clone analyzed exhibited minor differences in nucleotide sequence, a change eliminating an *Alu*-1 site was common to all. Amino acid changes found in individual clones 1, 2, and 3 are indicated by superscript. CHO, potential N-linked glycosylation sites.

lysine at position 211, asparagine to lysine at position 312, cysteine to glutamic acid at position 338, valine to alanine at position 346, and glutamic acid to lysine at position 350) were all located within the variable domain (amino acid positions 138 to 361). In this variable region, only the substitutions at positions 346 and 350 have not been documented in the Wyoming strain or pony-passaged isolates of the PR strain (35–37). Both of these amino acid substitutions are located in the C-terminal portion of the variable domain in a region postulated to form an α-helical structure (3).

Nucleotide sequence analysis of the extracellular domain of the FDD virus TM protein did not reveal any dramatic changes from the parental PR strain. However, unlike individual molecular clones containing FDD virus SU sequences, which showed uniformity in amino acid substitutions compared with the PR strain, the results from three TM-containing clones sequenced produced slight differences. Although each clone contained three nucleotide changes compared with published PR sequences (44), only one of these was common to all. This common change did not affect amino acid content but did eliminate an *Alu*-1 site (Fig. 4). As for the remaining two nucleotide substitutions present in each clone, only one resulted in a change in amino acid content compared with the parental PR strain. However, in each case, these amino acid substitutions appeared to be conservative (Fig. 4). The extracellular domain of the TM protein was considered unlikely to determine the neutralization sensitivity of the FDD virus strain because of the lack of consistent or significant amino acid substitutions compared with the parental PR strain.

The role played in sensitivity to neutralizing antibody by the amino acid changes in the SU protein was investigated by substituting a 1,173-bp *Bam*HI-*Hin*dIII fragment from the FDD ($FDD_{B\text{-}H}$) virus strain into an infectious molecular clone of EIAV. This fragment encompassed all the amino acid changes in the SU protein found in the FDD virus SU protein

TABLE 2. Neutralization titers of serum samples from PR-infected ponies and mouse MAbs against chimeric 44-1/FDD viruses[a]

Virus strain	Log_{10} neutralizing titer			
	Pony 78	Pony 918	gp90-E	gp90-A
PR	<1	<1	<1	<1
44-1	<1	<1	<1	<1
FDD	4.0	3.5	3.0	<1
44-1/$FDD_{B\text{-}H}$ 8	4.5	4.0	ND[b]	<1
44-1/$FDD_{B\text{-}H}$ 13	3.5	3.0	3.0	<1
44-1/$FDD_{B\text{-}H}$ 21	4.5	3.5	3.5	<1

[a] The virus dose was 10^2 $TCID_{50}$. MAb gp90-E binds to the SU protein of EIAV and neutralizes viral infectivity, whereas MAb gp90-A shows binding reactivity only with the SU protein (3, 17).
[b] ND, not done.

with the exception of the valine to isoleucine substitution at position 420.

Hybrid molecular clones (designated 44-1/$FDD_{B\text{-}H}$) were constructed and transfected into FDD cell cultures. The SU coding region from one of these clones was sequenced to ensure that it contained all the amino acid substitutions found in the FDD virus strain. It had been determined previously that transfection of 44-1 proviral DNA in FDD cells, followed by a single passage of the resultant virus in this cell line, did not increase its sensitivity to neutralization relative to transfection and passage in FEK cells (10). Therefore, stocks of 44-1/$FDD_{B\text{-}H}$ chimeric viruses for all subsequent experiments were prepared in FDD cells by a single passage of viruses produced as a result of transfection. It should be noted that chimeric 44-1/$FDD_{B\text{-}H}$ viruses did not produce accelerated cytopathic effects in FDD cells, indicating that this phenotypic trait did not map to SU sequences alone.

Viral stocks from three independently derived 44-1/$FDD_{B\text{-}H}$ infectious molecular clones (designated 8, 13, and 21) were tested in neutralization assays against a panel of equine serum samples obtained from two PR-infected ponies (78 and 918). In addition to equine serum samples, the 44-1/$FDD_{B\text{-}H}$ chimeric viruses were also tested against murine MAbs directed against epitopes A and E on the SU protein (17). MAb gp90-A is nonneutralizing and directed against an epitope in the conserved N-terminal region of the SU protein (3, 17), whereas MAb gp90-E is directed against an epitope in the putative PND (3) and is capable of neutralizing viral infectivity (17). The panel of postinfection equine serum samples used in these experiments did not neutralize the PR strain at the starting dilution of 10^{-1}, whereas the same serum samples neutralized the FDD virus strain at dilutions ranging from 10^{-3} to 10^{-4} (Table 2). Viruses produced from infectious molecular clone 44-1 were also not neutralized by the equine serum samples at the starting dilution of 10^{-1}. However, substitution of the *Bam*HI-*Hin*dIII fragment from the FDD virus strain dramatically changed the neutralization profile (Table 2). Chimeric viruses derived from 44-1/$FDD_{B\text{-}H}$ molecular clones produced neutralization profiles virtually identical to those obtained with the FDD virus strain (Table 2). The results with MAb gp90-E were analogous to those seen with postinfection serum samples, i.e., substitution of the $FDD_{B\text{-}H}$ fragment enhanced the neutralizing sensitivity of 44-1 to a level equivalent to that with the FDD virus strain (Table 2). As expected, none of the virus strains were neutralized by MAb gp90-A (Table 2).

DISCUSSION

As stated above, the weak neutralizing antibody responses seen in lentivirus-infected animals could be caused by poor

immunogenicity of neutralizing epitopes or by innate resistance of these viruses to the action of such antibodies. However, in the experiments described here, the results of neutralization assays with the FDD virus suggested that neutralizing epitopes on the PR strain of EIAV are not intrinsically poor immunogens but can stimulate relatively high neutralizing antibody titers in ponies by 100 days p.i. The fact that these high titers were not observed in neutralization assays with the PR strain is consistent with the hypothesis that the virus is resistant to the action of these antibodies. This conclusion is dependent on whether the in vitro assay accurately reflects the situation in vivo and assumes that mutations in the FDD virus strain do not result in the conversion of nonneutralizing to neutralizing determinants. However, it is tempting to speculate that the resistance to neutralizing antibody is an important survival characteristic compensating for the highly immunogenic properties of the EIAV SU protein. In fact, the pronounced immunogenicity of this protein may provide a selective advantage to the virus. In recent studies, we have shown that the SU protein of EIAV contains determinants capable of stimulating host responses which enhance viral replication and disease progression (53).

The experiments with the 44-1 and 44-1/FDD_{B-H} chimeric viruses provide substantial evidence that the sensitivity of the FDD virus strain to neutralization by antibody is conferred by amino acid substitutions in the SU protein and not by epigenetic phenomena under host cell control. The virus stock designated the FDD virus strain was derived from the PR strain by serial passage in FDD cells (39). Therefore, it is probable that some or all of the amino acid substitutions observed in the FDD virus SU protein arose because of selective pressures exerted by the FDD cell line. These pressures could cause mutations to occur during passage or, alternatively, could result in the selection of a distinct subpopulation which was present in the PR stock. Host cell selection pressures affecting viral antigenicity have been described for influenza A and B viruses, for which the mechanism appears to be selection of subpopulations (11, 22, 42, 43, 50). In addition, host cell selection pressures have also been described for HIV; the resistance of primary isolates of this virus to neutralization by soluble CD4 molecules can be significantly reduced by extended passage in continuous cell lines (13, 52). Furthermore, it has recently been demonstrated that clinical isolates and certain laboratory strains of HIV are much more sensitive to neutralization by human antisera and MAbs when grown in H9 cells than in peripheral blood mononuclear cell cultures (49). However, unlike the gradual process which was responsible for the loss of resistance to neutralization by soluble CD4 molecules (13, 52), changes in sensitivity to neutralization by antibody occurred within three passages and could be reversed with equal rapidity. Although these rapid changes suggest that epigenetic phenomena may be responsible for cell-specific differences in antigenicity, amino acid substitutions were detected between the SU proteins of the same HIV isolates grown in either H9 and peripheral blood mononuclear cell cultures. Unfortunately, the potential significance of these amino acid substitutions to neutralization sensitivity has yet to be determined (49). In EIAV, the number of passages in FDD cells required to enhance sensitivity to neutralizing antibody is under investigation. Furthermore, investigations are under way to determine if the sensitivity of the FDD virus strain can be reversed by passage in FEK cells. However, a single passage of the FDD virus strain in FEK cells, along with experiments in which molecular clone 44-1 was transfected and passaged in both FEK and FDD cells (10), indicates that sensitivity to neutralization in this EIAV system is not determined at the very earliest passage levels. Although amino acid substitutions in the SU protein were not sufficient to confer accelerated cytopathic effects, it is interesting that this trait was not fully manifested until the 18th passage of the PR strain in FDD cells (18). Therefore, this aspect of adaptation to growth in FDD cells appears to be a gradual process analogous to increased neutralization of HIV-1 by soluble CD4.

Amino acid substitutions in the SU protein of the FDD virus variant are consistent with the influence of host cell selection pressures. However, the molecular mechanism(s) and the stage(s) in the viral replication cycle at which such selection pressures are applied remain to be determined. In addition, it is not known if these selective effects reflect rather narrow differences between FDD and FEK cell types or species differences between cells of *Equus caballus* and *Equus asinus* origin. It is interesting that the live vaccine used extensively against equine infectious anemia in China was attenuated by passage in donkey leukocyte cultures (51). Therefore, at least under in vitro conditions, switching the growth of EIAV from cells of horse origin to cells of donkey origin may exert selection pressure resulting in phenotypic changes, ranging from attenuation of virulence to an increase in neutralization sensitivity. It is not known whether these changes are linked or whether similar changes could occur in vivo in donkeys infected with horse-derived strains of EIAV.

The role of the individual amino acid substitutions in the neutralization sensitivity of the FDD virus strain is now under investigation in this laboratory. Two of the amino acid substitutions (at positions 211 and 350) would result in a reversal of charge polarity, four (at positions 312, 338, 350, and 394) would introduce a charge, and three (at positions 194, 346 and 420) would have no effect on charge. However, analysis based on a number of predictive algorithms has indicated that the secondary structure of the FDD virus SU protein would have only minimal differences from a previously published model for the PR SU protein (3, 29). Furthermore, the amino acid substitutions in the FDD virus SU protein did not change the number of potential N-linked glycosylation sites. Compared with PR published sequences (44), the FDD virus strain contains an amino acid substitution in the PND (which is in the epitope recognized by the neutralizing MAb gp90-E), although preliminary evidence suggests that this change alone does not contribute significantly to sensitivity to neutralization. Infectious molecular clone 19-2 contains the same threonine-to-methionine substitution at position 194 in the PND as the FDD virus strain (37), and yet viruses derived from it are not more sensitive than viruses derived from 44-1 or the PR strain in neutralization assays (10). Therefore, it appears that most of the amino acid substitutions in the FDD virus SU protein are at linearly distant sites from known neutralizing epitopes. However, such linearly distant substitutions are likely to be capable of effecting changes in known neutralizing epitopes, such as the one recognized by MAb gp90-E, so that enhancement of neutralization can occur. This was not totally unexpected, as it has been well documented that amino acid substitutions at distant sites can have profound effects on the antigenicity and function of proteins from different viruses, including HIV (2, 6, 14, 40, 54, 56, 57).

In summary, the results presented here suggest that the PR strain has an innate resistance to the actions of neutralizing antibody which was altered when it was passaged in cells of donkey origin. The variant FDD virus strain has amino acid substitutions in the SU protein which are responsible for increases in sensitivity to neutralizing antibody.

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