

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

R. FRANK COOK,¹ SANDRA L. BERGER,¹ KEITH E. RUSHLOW,² JACQUELINE M. McMANUS,¹ SHEILA J. COOK,¹ SHARON HARROLD,³ MICHELLE L. RAABE,³ RONALD C. MONTELARO,³ AND CHARLES J. ISSEL^{1*}

Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546¹; Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261³; and Paravax Inc., Fort Collins, Colorado 80526²

Received 11 August 1994/Accepted 18 November 1994

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

* Corresponding author. Mailing address: Department of Veterinary Science, 108 Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099. Phone: (606) 257-1710. Fax: (606) 257-8542. Electronic mail address: VSCISSEL@UKCC.UKY.EDU.

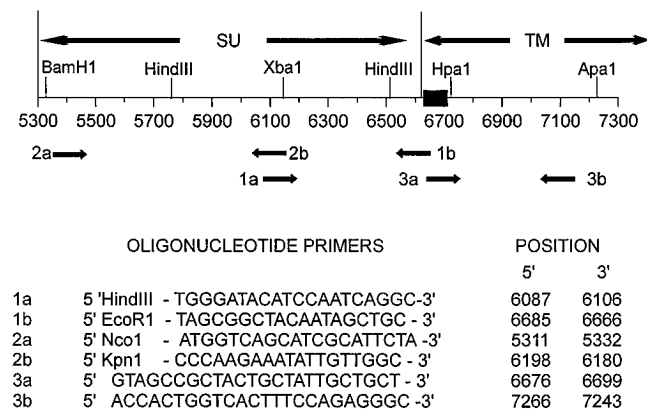


FIG. 1. Oligonucleotide primers used in PCR amplification of SU and TM (extracellular domain) coding sequences from the FDD virus variant of EIAV. Position numbering is according to the complete proviral sequence (GenBank accession number M16575). The black box area represents the fusion domain.

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 μ g/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 μ g of this DNA was added to a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each of the four

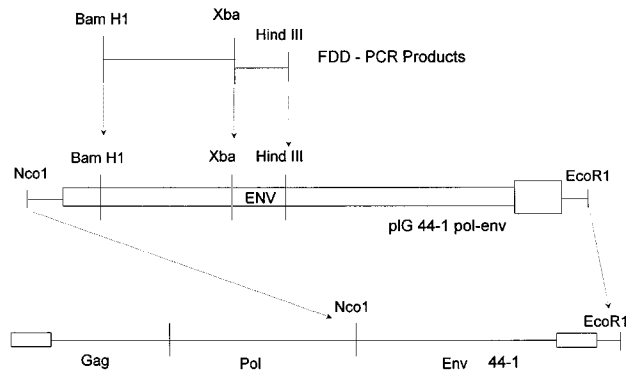


FIG. 2. Construction of a chimeric virus containing FDD virus strain SU protein sequences in the 44-1 infectious molecular clone of EIAV. Primer pairs 2a and 2b and 1a and 1b were used to amplify the region of the FDD virus genome encoding the SU protein. These PCR products were digested with *Bam*HI and *Xba*I or *Xba*I and *Hind*III and ligated to form a 1,173-bp fragment, which was inserted into *Bam*HI-*Hind*III-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-H}. In the final manipulation, the *Nco*I-*Eco*RI fragment from pLG44-1*env*/FDD_{B-H} was used to replace an equivalent fragment from 44-1 to create 44-1/FDD_{B-H}.

dioxynucleoside triphosphates, 0.25 μ M 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 μ l with distilled H₂O. 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 *Hinc*II-digested pUC13. For nucleotide sequencing purposes, the 610-bp fragment produced from the primer pair 1a and 1b reaction was also digested with *Hind*III, and the resultant 430-bp *Hind*III-*Hind*III fragment was cloned into pUC13 which had been digested with *Hind*III 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 *Hind*III 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-*Hind*III (350 bp) FDD proviral DNA fragment which had been amplified by using the 1a and 1b primers (Fig. 1). This FDD virus *Bam*HI-*Hind*III fragment was then inserted into *Bam*HI-*Hind*III-digested pLG44-1*pol-env* to generate pLG44-1*env*/FDD_{B-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-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 10² TCID₅₀.

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-*Hind*III 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*-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 [TCID₅₀] 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 NaHCO₃, 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 (log₁₀ 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 Na₂S₂O₃, 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'-tetramethylbenzidine (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

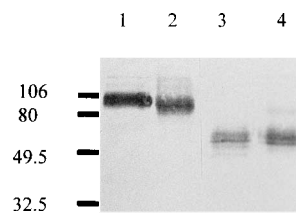


FIG. 3. Immunoblot analysis of the SU protein from purified preparations of the PR and FDD virus strains. Both viral preparations were examined before and after treatment with *N*-glycosidase F and *O*-glycosidase. Following electrophoretic transfer from a polyacrylamide gel to a nitrocellulose filter, 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} TCID₅₀ with serum from pony 918, the log₁₀ 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

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.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (RO1AI25850), the University of Kentucky (grant 4-32107), the Kentucky Agricultural Experiment Station, and the Lucille P. Markey Charitable Trust and by NIH Training Grant 1-T32-AI-07487-01 to S.H.

REFERENCES

- Arendrup, M., C. M. Nielson, J.-E. S. Hansen, L. Mathiesen, B. O. Lindhardt, E. Scheibel, and J. O. Nielson. 1992. Neutralizing antibodies against two HIV-1 strains in consecutively collected serum samples: cross neutralization and association to HIV-1 related disease. *Scand. J. Infect. Dis.* **24**:21–28.
- Back, N. K. T., L. Smit, M. Schutten, P. L. Nara, M. Tersmette, and J. Goudsmit. 1993. Mutations in human immunodeficiency virus type 1 gp41 affect sensitivity to neutralization by gp120 antibodies. *J. Virol.* **67**:6897–6902.
- Ball, J. M., K. E. Rushlow, C. J. Issel, and R. C. Montelaro. 1992. Detailed mapping of the antigenicity of the surface unit glycoprotein of equine infectious anemia virus by using synthetic peptide strategies. *J. Virol.* **66**:732–742.
- Barnett, D. 1972. Heat inactivation of equine infectious anemia virus in equine serum. *Proc. U.S. Anim. Health Assoc.* **76**:231–236.
- Bigon, C., and P.-M. Martin. 1992. Improved sequencing of “mini-prep” double stranded templates using a multiwell microplate system. *Biotechniques* **13**:194–198.
- Bolwell, C., N. R. Parry, and D. J. Rowlands. 1992. Comparison between *in vitro* neutralization titers and *in vivo* protection against homologous and heterologous challenge induced by vaccines prepared from two essentially serologically distinct variants of foot-and-mouth disease virus, serotype A22. *J. Gen. Virol.* **73**:727–731.
- Chen, C., and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient system for stably transforming cells with plasmid DNA. *Biotechniques* **6**:632–638.
- Clabough, D. L. 1990. Equine infectious anemia: the clinical signs, transmission, and diagnostic procedures. *Vet. Med.* **85**:1007–1019.
- Cook, R. F., S. J. Cook, and C. J. Issel. 1992. A non-radioactive micro-assay for released reverse transcriptase activity of a lentivirus. *Biotechniques* **13**:380–386.
- Cook, R. F., and C. J. Issel. Unpublished data.
- Cook, R. F., J. A. Mumford, A. Douglas, and J. M. Wood. 1988. The influence of host cell on the antigenic properties of equine-2 influenza A viruses, p. 60–65. *In Equine Infectious Diseases V: Proceedings of the Fifth International Conference on Equine Infectious Diseases.*
- Cunningham, T. P., R. C. Montelaro, and K. E. Rushlow. 1993. Lentivirus envelope sequences and proviral genomes are stabilized in *Escherichia coli* when cloned into low copy-number plasmid vectors. *Gene* **124**:93–98.
- Daar, E. S., X. L. Li, T. Moudgil, and D. D. Ho. 1990. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc. Natl. Acad. Sci. USA* **87**:6574–6578.
- Fujita, K., J. Silver, and K. Peden. 1992. Changes in both gp120 and gp41 can account for increased growth potential and expanded host range of human immunodeficiency virus type 1. *J. Virol.* **66**:4445–4451.
- Goudsmit, J., C. Deboucq, R. H. Melen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek. 1988. Human immunodeficiency virus type 1 neutralizing epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proc. Natl. Acad. Sci. USA* **85**:4478–4482.
- Huso, D. L., O. Narayan, and G. Hart. 1988. Sialic acids on the surface of caprine arthritis-encephalitis virus define the biological properties of the virus. *J. Virol.* **62**:1974–1980.
- Hussain, K. A., C. J. Issel, K. L. Schnorr, P. M. Rwambo, and R. C. Montelaro. 1987. Antigenic analysis of equine infectious anemia virus (EIAV) variants by using monoclonal antibodies: epitopes of glycoprotein gp90 of EIAV stimulate neutralizing antibodies. *J. Virol.* **61**:2956–2961.
- Issel, C. J. Unpublished data.
- Issel, C. J., D. W. Horohov, D. F. Lea, W. V. Adams, Jr., S. D. Hagius, J. M. McManus, A. Allison, and R. C. Montelaro. 1992. Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. *J. Virol.* **66**:3398–3408.
- Issel, C. J., K. E. Rushlow, L. D. Foil, and R. C. Montelaro. 1988. A perspective on equine infectious anemia with an emphasis on vector transmission and genetic analysis. *Vet. Microbiol.* **17**:251–286.
- Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews. 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci. USA* **86**:6768–6772.
- Katz, J. M., C. W. Naeve, and R. G. Webster. 1987. Host-cell mediated variation in H3N2 influenza virus. *Virology* **156**:386–395.
- Kennedy-Stoskopf, S., and O. Narayan. 1986. Neutralizing antibodies to visna lentivirus: mechanisms of action and possible role in persistence. *J. Virol.* **59**:37–44.
- Kono, Y., K. Kobayashi, and Y. Fukunaga. 1973. Antigenic drift of equine infectious anemia virus in chronically infected horses. *Arch. Virol.* **41**:1–10.
- Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand. 1988. Using mini-prep plasmid DNA for sequencing double stranded template with sequenase. *Biotechniques* **6**:544–546.
- Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **265**:10373–10382.
- Letvin, N. L. 1991. Immune responses in lentivirus-infected animals. *Curr. Opin. Immunol.* **3**:559–563.
- Malmquist, W. A., D. Barnett, and C. S. Becvar. 1973. Production of equine infectious anemia antigen in a persistently infected cell line. *Arch. Virol.* **42**:361–370.
- Montelaro, R. C. Unpublished data.
- Montelaro, R. C., N. Lohrey, B. Parekh, E. W. Blakeney, and C. J. Issel. 1982. Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. *J. Virol.* **42**:1029–1038.
- Narayan, O., J. E. Clements, S. Kennedy-Stoskopf, D. Sheffer, and R. Royal. 1987. Mechanism of escape of visna virus from immunological control. *Contrib. Microbiol. Immunol.* **8**:60–78.
- Narayan, O., D. Sheffer, D. E. Griffin, J. E. Clements, and J. Hess. 1984. Lack of neutralizing antibody to caprine arthritis-encephalitis lentivirus in persistently infected goats can be overcome by immunization with inactivated *Mycobacterium tuberculosis*. *J. Virol.* **49**:349–355.
- O'Rourke, K., L. E. Perryman, and T. C. McGuire. 1988. Antiviral, anti-glycoprotein and neutralizing antibodies in foals with equine infectious anemia virus. *J. Gen. Virol.* **69**:667–674.
- Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-coded synthetic peptides. *Proc. Natl. Acad. Sci. USA* **85**:1932–1936.
- Parekh, B., C. J. Issel, and R. C. Montelaro. 1980. Equine infectious anemia virus, a putative lentivirus, contains polypeptides analogous to prototype-C oncornaviruses. *Virology* **107**:520–525.
- Payne, S. L., F.-D. Fang, C. P. Liu, B. R. Dhruva, P. Rwambo, C. J. Issel, and R. C. Montelaro. 1987. Antigenic variation and lentivirus persistence: variations in envelope gene sequences during EIAV infection resemble changes reported for sequential isolates of HIV. *Virology* **161**:321–331.
- Payne, S. L., P. J. Rausch, K. E. Rushlow, R. C. Montelaro, C. J. Issel, M. Flaherty, S. Perry, D. Sellon, and F. Fuller. 1994. Characterization of infectious molecular clones of equine infectious anemia virus. *J. Gen. Virol.* **75**:425–429.
- Perry, S. T., M. T. Flaherty, M. J. Kelley, D. L. Clabough, S. R. Tronick, L. Coggins, L. Whetter, C. R. Lengel, and F. Fuller. 1992. The surface envelope protein gene region of equine infectious anemia virus is not an important determinant of tropism *in vitro*. *J. Virol.* **66**:4085–4097.
- Rasty, S., B. R. Dhruva, R. L. Schiltz, D. S. Shih, C. J. Issel, and R. C. Montelaro. 1990. Proviral DNA integration and transcriptional patterns of equine infectious anemia virus during persistent and cytopathic infections. *J. Virol.* **64**:86–95.
- Reitz, M. S., Jr., C. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff. 1988. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. *Cell* **54**:57–63.
- Robert-Guroff, M., M. Brown, and R. C. Gallo. 1985. HTLV-III neutralizing antibodies in patients with AIDS and AIDS-related complex. *Nature (London)* **31**:72–74.
- Robertson, J. S., J. S. Bootman, R. Newman, J. S. Oxford, R. S. Daniels, R. G. Webster, and G. C. Schild. 1987. Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A (H1N1) virus. *Virology* **160**:31–37.
- Robertson, J. S., C. W. Naeve, R. G. Webster, J. S. Bootman, R. Newman, and G. C. Schild. 1985. Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. *Virology* **143**:166–174.
- Rushlow, K. E., K. Olsen, G. Stiegler, S. L. Payne, R. C. Montelaro, and C. J. Issel. 1986. Lentivirus genomic organization: the complete nucleotide sequence of the env gene region of equine infectious anemia virus. *Virology* **155**:309–321.
- Rwambo, P. M., C. J. Issel, W. V. Adams, Jr., K. A. Hussain, M. Miller, and R. C. Montelaro. 1990. Equine infectious anemia virus (EIAV): humoral responses of recipient ponies and antigenic variation during persistent infection. *Arch. Virol.* **111**:199–212.
- Rwambo, P. M., C. J. Issel, K. A. Hussain, and R. C. Montelaro. 1990. *In vitro* isolation of a neutralization escape mutant of equine infectious anemia virus (EIAV). *Arch. Virol.* **111**:275–280.
- Salinovich, O., S. L. Payne, R. C. Montelaro, K. A. Hussain, C. J. Issel, and K. L. Schnorr. 1986. Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. *J. Virol.* **57**:71–80.

48. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Sawyer, L. S. W., M. T. Wrin, L. Crawford-Mikszka, B. Potts, Y. Wu, P. A. Weber, R. D. Alfonso, and C. V. Hanson.** 1994. Neutralization sensitivity of human immunodeficiency virus type 1 is determined in part by the cell in which the virus is propagated. *J. Virol.* **68**:1342–1349.
50. **Schild, G. C., J. S. Oxford, J. C. de Jong, and R. G. Webster.** 1983. Evidence for host-cell selection of influenza virus antigenic variants. *Nature (London)* **303**:706–709.
51. **Shen, R.** 1983. Development and use of an equine infectious anemia donkey leucocyte attenuated vaccine, p. 34–53. *In* Proceedings of the International Symposium on Immunity to Equine Infectious Anemia. Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Beijing.
52. **Turner, S., R. Tizard, J. DeMarinis, R. B. Pepinsky, J. Zullo, R. Schooley, and R. Fisher.** 1992. Resistance of primary isolates of human immunodeficiency virus type 1 to neutralization by soluble CD4 is not due to lower affinity with viral envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. USA* **89**:1335–1339.
53. **Wang, S. Z.-S., K. E. Rushlow, C. J. Issel, R. F. Cook, S. J. Cook, M. L. Raabe, Y.-H. Chong, L. Costa, and R. C. Montelaro.** 1994. Enhancement of EIAV replication and disease by immunization with a baculovirus-expressed recombinant envelope surface glycoprotein. *Virology* **199**:247–251.
54. **Watkins, B. A., M. S. Reitz, Jr., C. A. Wilson, K. Aldrich, A. E. Davis, and M. Robert-Guroff.** 1993. Immune escape by human immunodeficiency virus type 1 from neutralizing antibodies: evidence for multiple pathways. *J. Virol.* **67**:7493–7500.
55. **Weiss, R. A., P. R. Clapman, R. Cheingsong-Popov, A. G. Dalgleish, C. A. Carne, I. V. D. Weller, and R. S. Tedder.** 1985. Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. *Nature (London)* **316**:69–72.
56. **Wilson, C., M. S. Reitz, Jr., K. Aldrich, P. J. Klasse, J. Blomberg, R. C. Gallo, and M. Robert-Guroff.** 1990. The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute the neutralization epitope. *J. Virol.* **64**:3240–3248.
57. **Wyatt, R., M. Thali, S. Tilley, A. Pinter, M. Posner, D. Ho, J. Robinson, and J. Sodroski.** 1992. Relationship of human immunodeficiency virus type 1 gp120 third variable loop to a component of the CD4 binding site in the fourth conserved region. *J. Virol.* **66**:6997–7004.