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Combined amino acid mutations occurring in the envelope closely correlate with pathogenicity of EIAV

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Summary. The Chinese equine infectious anemia virus (EIAV) donkey-leukocyte attenuated vaccine (DLV) provides a unique natural model system to study the attenuation mechanism and immunological control of lentivirus replication. Critical consensus mutations were identified between virulent Chinese EIAV strains and vaccine strains. Based on a full-length infectious clone of EIAV vaccine strain pLGFD3, two molecular clones, mFD5-4-7 and mFD7-2-11, were successfully constructed, in which 4 and 6 critical consensus mutations in the env gene of the vaccine strain were point-mutated to the wild-type sequence, respectively by an overlap PCR mutagenesis strategy. The infectivity, virulence, and pathogenesis of the constructed clones were investigated *in vitro* using a reverse transcriptase assay, an indirect immunofluorescence assay, observation of cytopathogenic effect, and virion observation as well as *in vivo* by inoculation of animals with the resulting infectious clones. The pathogenic symptoms in horses inoculated with mFD7-2-11 were more severe than those inoculated with mFD5-4-7, whereas no pathogenic symptoms were detected in animals inoculated with their parental clone pLGFD3 strain. The results indicate that the consensus mutation residues of the env region involved in this study play significant roles in the virulence and pathogenicity of EIAV. This will contribute to the elucidation of the attenuating and protective mechanisms of the Chinese EIAV vaccine.

H. Liang and X. He contributed equally to this paper.

Introduction

Live attenuated lentivirus vaccines have been proven to be the most effective approach in driving a critical maturation of virus-specific humoral and cellular immune responses according to study results from animal lentivirus systems including equine infectious anemia virus (EIAV) for horse and simian immunodeficiency virus (SIV) for monkey [28, 33, 47, 51]. However, the absence of suitable animal models for human immunodeficiency virus type 1 (HIV-1) vaccine development has been one of the most frustrating hurdles for evaluating the efficacy and protective immune mechanism of potential vaccines. EIAV is a macrophage-tropic lentivirus that can cause acute, chronic and asymptomatic infection in infected horses, depending on different levels of viral replication and host immune status [38]. Interestingly, most chronically infected horses survive an inapparent carrier phase after recurring cycles of fever, anemia, weight loss, and thrombocytopenia [7, 45]. This provides an excellent model for identifying the critical mechanism of lentivirus immune protection and indicates the potential of developing an effective preventive vaccine for HIV exposure.

In the 1970s, a Chinese EIAV donkey-leukocyte attenuated vaccine (DLV) was developed by a series of *in vitro* passages of EIAV pathogenic strain D510, which was obtained from *in vivo* passage with the wild type EIAV LN40 strain [46]. The near full-length sequences of LN40, DLV and FDDV were patented (Patent No. CN99105852.6 and US6987020B1) and applied patent (Patent No. CN99127532.2). The vaccine proved to be effective, with 79% of vaccinated horses resisting challenge by homogeneous and heterogeneous virulent EIAV [46]. Based on DLV, another fetal donkey dermal cell-adapted vaccine FDDV was developed by passage of DLV on fetal donkey dermal cells for 15 generations. FDDV proved to be more effective than DLV, with 95% protection for horses [48]. The differences in genetic and antigenic properties between the virulent strains and vaccine strains provided a useful and novel lentivirus system for research on the mechanism of immune protection and vaccine development of lentivirus, including HIV.

It was considered that *env* gene was involved in pathogenesis, as implicated in some lentivirus diseases [3, 4, 6, 16, 17, 37, 41, 49]. Furthermore, most differences between virulent and avirulent or vaccine strains were located in the *env* region of EIAV genome, and these differences often resulted in changes of N-linked glycosylation sites [31, 32, 35]. Based on sequence analysis of two Chinese virulent strains (LN40 and D510) and two vaccine strains (DLV and FDD), 10 consensus mutational sites had been confirmed in the *env* region. In this report, we explore whether or not these nucleotide sites are associated with pathogenicity in EIAV.

Infectious molecular clone technology is a powerful tool for investigating the genetic elements responsible for virulence and pathogenicity because it allows the introduction of point mutations, deletions, and insertions in the viral genome. Production of mutant molecular clones has already yielded insights into viral sequences associated with pathogenicity in EIAV and other lentivirus [8, 13, 14, 21, 26, 29]. In this report, based on the backbone of a full-length infectious molecular clone pLGFD3 of vaccine strains, two infectious clones, mFD5-4-7 and mFD7-2-11, were constructed with consensus mutational sites of the *env* gene of the vaccine

strains point-mutated to the wild-type strain. The infectivity, virulence, and pathogenesis of the resulting molecular clones were investigated both *in vitro* and *in vivo*.

Material and methods

Computer-assisted sequence analysis

The Clustal W program was used for alignment of protein primary structure. The results were edited by Genedoc software.

Plasmid, virus stock, and cell culture

Plasmid pLGFD3, a full-length infectious molecular clone derived from Chinese EIAV fetal donkey dermal cell-adapted vaccine (FDDV) strain, was constructed in the low-copy-number plasmid pLG338 (provided by NIH AIDS research and reference reagent program) [24].

Fetal donkey dermal cells (FDD) were maintained in minimal essential medium (MEM) with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU of penicillin per ml and 100 μg of streptomycin per ml. Donkey primary lymphocyte (DL) cultures were prepared from heparinized peripheral blood as described for equine MDM (monocyte-derived macrophage) cultures [43]. Briefly, supernatant plasma (containing mononuclear cells) was isolated from fresh heparinized donkey whole blood after allowing it to stand at room temperature for 30 min. Following several washes in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), cells were seeded in α-MEM (minimal essential medium, Gibco BRL) with 10% heat-inactivated horse serum (Sigma) on each gelatin-coated (Sigma)- and plasma-coated (Corning) tissue culture dish (150 cm^2) and incubated overnight at 37 °C with 6% CO_2. After 24 h, the nonadherent and loosely adherent cells were removed by repeated vigorous washing with α-MEM. The adherent cells were detached with 5 mM EDTA in α-MEM with 10% heat-inactivated horse serum and seeded into 48-well plates (Corning) at a density of 10^5 cells per well.

DNA mutagenesis and construction of mutant molecular clones

The point-mutant molecular clones were constructed based on plasmid pLGFD3 (Fig. 2). Mutations were introduced using Quik Change Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacture's protocol. Primers used for mutagenesis are listed in Table 1. Mutated segments were digested with Nco I and Nru I and substituted for the corresponding segments of pLGFD3 to generate full-length molecular clones with 4 amino acids changed for mFD5-4-7 and 6 for mFD7-2-11.

Transfection of FDD cell culture and production of virus

Plasmid DNA preparations from each individual full-length molecular clone of EIAV were purified using a Midiprep kit (Qiagen, Germany). FDD cells were seeded in 6-well plates (2×10^5 cells per well). When the FDD cells were 50 to 70% confluent, the cells were transfected with 4.0 μg of plasmid DNA using the DOTAP Liposomal Transfection System (Roche, Germany) according to the manufacturer's recommendations. The first generation of virus supernatant was collected at 14 days after transfection and passage on FDD cells was continued for 4 generations. Since EIAV is a macrophage-tropic lentivirus, the 5th generation of virus from FDD cells was then passaged on DL cells for another 5 generations to adapt the macrophages. Cell culture supernatants were collected when a cytopathogenic effect (CPE) appeared in the cells (about 10 days on FDD cells and 4 days on DL cells). Aliquots of the cell culture supernatants were stored at −80 °C for the reverse transcriptase activity assay using the reverse transcriptase assay colorimetric kit (Roche, Germany) as recommended by the manufacturer.

Indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) was performed to confirm expression of viral protein. Freshly grown monolayers of FDD cells were infected with pLGFD3, mFD5-4-7, and mFD7-2-11 strains, respectively, and cultured at 37 °C concurrently with uninfected cells serving as negative controls. When CPE appeared (usually 10 days postinfection), the cells were digested and a cell smear was prepared and fixed with ice-cold acetone for 30 min at −20 °C. After two washes with PBS, cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After washing a further three times, cells were incubated with blocking solution (containing 5% non-fat milk, 5% fetal calf serum, and 0.025% Tween in PBS) for 1 h at 37 °C in a humid atmosphere. Smears were then washed three times with PBS and incubated with EIAV positive serum (1:200) in blocking solution in a moist chamber at room temperature for 60 min. Subsequently, cells were washed three times with PBS and incubated with FITC-labeled rabbit antihorse IgG (1:3000) (Sigma) in blocking solution for 1 h. Slides were counterstained with 0.01% Evans blue and washed three times in PBS and a fourth time in double-distilled water. Afterwards, the cells were covered with glycerol and observed by fluorescence microscopy (Olympus, Tokyo, Japan).

Observation of virion formation by electron microscopy

When DL cells demonstrated CPE after infection (usually 4 days later), cells were collected and prepared for observation by negative-contrast electron microscopy (JEM-1200-EX; JEOL).

Minimal infectious dose analysis

The minimal infectious doses (MID) of the virus stocks were determined by performing serial ten-fold dilutions with α-MEM. 100 μl of each dilution was inoculated onto fresh DL cell cultures in 24-well dishes and cultured for 4 days. Aliquots of the DL culture supernatants were then collected and analyzed by reverse transcriptase activity assay. The MID of each virus stock was defined as the reciprocal of the highest dilution that still exhibited positive reverse transcriptase activity in DL culture supernatants.

Experimental infections

Six horses (horses 15, 17, 32–34, and 39) used in this study were thoroughbreds and serum samples from all horses were tested twice by the agar gel immunodiffusion test (VMRD, USA) before the experiment to ensure that they were seronegative for EIAV [11]. They were housed in screened box stalls to exclude hematophagous insects. All animal handling protocols were confirmed by the Animal Management Committee of Chinese Harbin Veterinary Research Institute. Six horses were randomly divided into three groups and were inoculated hypodermically with 10^5 minimal infectious doses (MID) of pLGFD3 (horses 15 and 17), mFD5-4-7 (horses 32 and 33), and mFD7-2-11 (horses 34 and 39) virus. Rectal temperature and clinical status were recorded daily. Samples of whole blood and plasma were collected at regular intervals (0, 15, 30, 45, 60, 75, 90, 120, 150, and 180 days post infection) and during each febrile episode (rectal temperature ≧39 °C; platelets <100,000/μl blood) for assays of platelets and level of viral replication. Platelet numbers were determined using the Nihon Kohden MEK-6318K celltac hematology analyzers (Nihon Kohden corp.).

Viral RNA purification

Viral RNA was isolated from 140 μl EDTA anticoagulated plasma using a QIAamp Viral RNA kit (Qiagen, Germany) and treated with DNase I on a spin column (DNase 1 set;

Qiagen) according to the manufacturer's instructions. RNA was eluted in 60 μl AVE buffer and analyzed immediately.

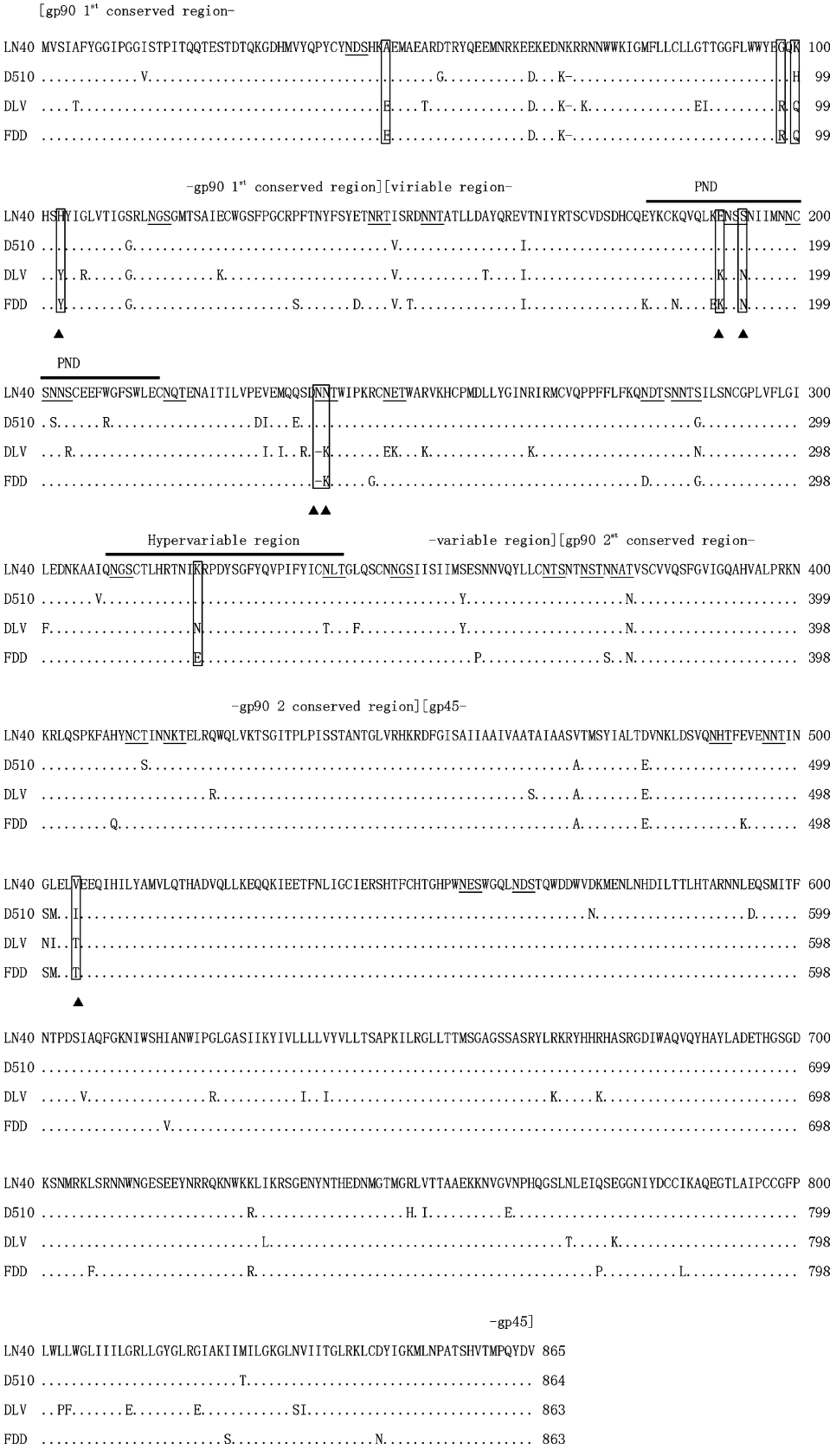
Quantitation of plasma virus RNA using TaqMan probe

Real-time RT-PCR was used to determine plasma viral load by amplification of a 66-bp segment of the FDD gag gene. The following oligonucletides were used for real-time RT-PCR: forward primer, 5′CAG ATT GCT GTC TCA GAT AAA 3′(nt 1344–1364); reverse primer, 5′GTG TCT GTC AGG AAT TTA GTT3′ (nt 1389–1409); and TaqMan probe (Applied Biosystems Inc.), 5′FAM-TCA GCC GGA TGT CCC TCA CT-TAMRA 3′ (nt 1366–1385). An RNA standard template was made by cloning pLGFD3 nt 1144–1533 into the PGEM T-easy vector (Promega), followed by in vitro transcription of the Sal I-linearized plasmid using the RiboMax Large-Scale RNA Production System-T7 (Promega). After transcription, RNA was DNase I treated and purified using an RNeasy Mini Kit (Qiagen). Integrity of the RNA transcripts was monitored by analysis on 1.2% agarose-formaldehyde gels and the copy numbers were determined following measurement of OD_{260}. Finally, the RNA transcripts were serially diluted in nuclease-free water containing 800 U/ml RNasin (Promega) and 30 μg/ml tRNA (Invitrogen) as a carrier and stored in aliquots at −80 °C until used. Real-time RT-PCR was performed using a QuantiTect^{TM} Probe RT-PCR Kit (Qiagen) in 50 μl reactions containing 25 μl 2 × RT-PCR Master Mix, 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 μM Taqman probe, 0.5 μl RT mix, 10 μl RNA sample or RNA standard. Reactions were performed in triplicate using an ABI Prism 7700 sequence detection system (Perkin-Elmer). Cycling conditions were 30 min at 50 °C, 15 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline as determined between cycles 3 and 15). Standard curves produced according to that CT value were proportional to the log of the starting amount of nucleic acids. The analysis of data was undertaken using Sequence Detection Software Version 1.9 (Perkin-Elmer).

Results

Comparison of primary amino acid sequences and confirmation of critical consensus mutations of the env gene between Chinese EIAV virulent and vaccine strains

To confirm the critical consensus mutations in the *env* gene between two virulent strains, LN40 and D510, and two vaccine strains, DLV and FDD, amino acid sequences of the four strains were compared and aligned. As shown in Fig. 1, ten critical consensus mutative sites were identified with nine of them in gp90 and one in gp45. Four of nine mutations, residues 46 (Ala → Glu), 97 (Gly → Arg), 99 (Lys → Gln), and 102 (His → Tyr), were located in the first conserved region of gp90. In the variable region of gp90, two mutations at residues 189 (Glu → Lys) and 192 (Ser → Asn) influenced the PND structure, and residue 192 also changed the N-glycosylation sites. Residues 235 (Asp → /) and 236 (Asp → Lys) were located just downstream of PND, and mutations of these two amino acids also influenced the potential N-glycosylation sites as residue 192 did. One mutation at residue 321 (Lys → Asn/Glu) occurred in the hypervariable region of gp90 [1, 40]. Only one mutation of residue 505 (Val/Ile → Thr) occurred in gp45, located in the third N helix of gp45 (Fig. 1).


[gp90 1st conserved region-
LN40 MVSIAFYGGIPGGISTPITQQTESTDTQKGDHMVYQPYCYNDSHKAEMAEARDTRYQEEMNRKEEKEDNKRRNNWWKIGMFLLCLLGTTGGFLWWYEGQK 100
D510 .....V.....G.....D...K-.....H 99
DLV ....T.....E....T.....D...K-.K.....EI.....R.Q 99
FDD .....E.....D...K-.....R.Q 99
-gp90 1st conserved region][viriable region-
PND
LN40 HSHYIGLVTIGSRLNGSGMTSAIECWGSFPGCRPFTNYFSYETNRTISRDNNTATLLDAYQREVTNIYRTSCVDSDHCQEYKCKQVQLKENSSNIIMNNC 200
D510 .....G.....V.....I..... 199
DLV ..Y..R.....G.....K.....V.....T....I.....K..N..... 199
FDD ..Y.....G.....S.....D....V.T.....I.....K...N....EK..N..... 199
PND
LN40 SNNSCEEFWGFSWLECNQTENAITILVPEVEMQQSDNNTWIPKRCNETWARVKHCPMDLLYGINRIRMCVQPPFFLFKQNDTSNNTSILSNCGPLVFLGI 300
D510 .S.....R.....DI...E.....G..... 299
DLV ...R.....I.I..R.-K.....EK...K.....K.....N..... 298
FDD .....-K.....G.....D.....G..... 298
Hypervariable region
-variable region][gp90 2st conserved region-
LN40 LEDNKAAIQNGSCTLHRTNIKRPDYSGFYQVPIFYICNLTGLQSCNNGSIISIIMSESNNVQYLLCNTSNTNSTNNATVSCVVQSFGVIGQAHVALPRKN 400
D510 .....V.....Y.....N..... 399
DLV F.....N.....T...F.....Y.....N..... 398
FDD .....E.....P.....S..N..... 398
-gp90 2 conserved region][gp45-
LN40 KRLQSPKFAHYNCTINNKTELRQWQLVKTSGITPLPISSTANTGLVRHKRDFGISAIIAAIVAATAIAASVTMSYIALTDVNKLDSVQNHTFEVENNTIN 500
D510 .....S.....A.....E..... 499
DLV .....R.....S.....A.....E..... 498
FDD .....Q.....A.....E.....K..... 498
LN40 GLELVEEQIHILYAMVLQTHADVQLLKEQQKIEETFNLIGCIERSHTFCHTGHPWNESWGQLNDSTQWDDWVDKMENLNHDILTTLHTARNNLEQSMITF 600
D510 SM..I.....N.....D..... 599
DLV NI..T..... 598
FDD SM..T..... 598
LN40 NTPDSIAQFGKNIWSHIANWIPGLGASIIKYIVLLLLVYVLLTSAPKILRGLLTTMSGAGSSASRYLRKRYHHRHASRGDIWAQVQYHAYLADETHGSGD 700
D510 699
DLV .....V.....R.....I..I.....K.....K..... 698
FDD .....V..... 698
LN40 KSNMRKLSRNNWNGESEEYNRRQKNWKKLIKRSGENYNTHEDNMGTMGRLVTTAAEKKNVGVNPHQGSLNLEIQSEGGNIYDCCIKAQEGTLAIPCCGFP 800
D510 .....R.....H.I.....E..... 799
DLV .....L.....T.....K..... 798
FDD .....F.....R.....P.....L..... 798
-gp45]
LN40 LWLLWGLIIILGRLLGYGLRGIAKIIMILGKGLNVIITGLRKLCDYIGKMLNPATSHVTMPQYDV 865
D510 .....T..... 864
DLV ..PF.....E.....E.....SI..... 863
FDD .....S.....N..... 863

In this study, we first selected consensus mutations located in PND or adjacent to PND for mutation analysis due to the importance of PND and N-linked glycosylation in the pathogenicity of EIAV. As a result, four of ten mutations displayed in the env region (102, 192, 235, and 236) were selected for mutations based on the backbone of infectious clone pLGFD3, derived from the Chinese attenuated EIAV vaccine. The resultant clone was designated mFD5-4-7. Once the infectivity of mFD5-4-7 was confirmed (described below), we added another two mutations, 189 and 505, for mutation analysis based on clone mFD5-4-7, with the resultant clone being designated mFD7-2-11. Residue 189 is located in PND and is covered by N-linked glycosylation to escape recognition of the host immune system in virulent strains. Residue 505 is in the third N helix of gp45 and is involved in membrane fusion.

Construction and replication of mutant molecular clones mFD5-4-7 and mFD7-2-11

Site-directed point-mutagenesis was done by two rounds of PCR using an overlap extension procedure, and point-mutated sites are marked with a triangle symbol in Fig. 2. Primers used for mutagenesis are listed in Table 1. The point-mutated molecular clones were sequenced twice to confirm the specified mutations. Digested mutant segments excised by restriction endonucleases Nco I and Nru I were substituted for the corresponding segments of pLGFD3 to generate two full-length molecular clones with 4 amino acids changed for mFD5-4-7 and 6 for mFD7-2-11.

The molecular clones mFD5-4-7 and mFD7-2-11 and their parental infectious clone pLGFD3 were transfected into FDD cells. Fourteen days later, the first generation of virus supernatants were collected and further passaged for 4 generations on FDD cells and then 5 generations on DL cells to adapt to macrophage tropism. Beginning with the 2nd generation on FDD cells, obvious CPE could be observed about 10 days postinoculation. CPE could be observed in all generations of DL

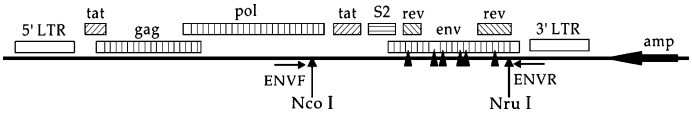


Fig. 2. Schematic diagram of EIAV vaccine clone pLGFD3. The triangle indicates the mutated sites in this study. Nco I and Nru I are unique restriction enzyme sites in the EIAV genome

Fig. 1. Comparison of deduced amino acid sequences of the *env* genes of Chinese virulent EIAV strains and vaccine strains. Amino acid residues that differ from the LN40 strain are indicated. A dot indicates that the amino acid is identical to the LN40 sequence and a dash indicates a deletion. The consensus mutations in vaccine strains are boxed. Potential N-linked glycosylation sites are underlined on the LN40 sequence. The hypervariable and PND regions are indicated by a thick black line. The triangle indicates mutational sites involved in this study

Table 1. Primer pairs for mutagenesis

Primer	Sequence (5′–3′)	Location
ENVF	TGTGTCCTTCATTGGCTATT	4594–4613
ENVR	GCCTTAAGGCAACAGTCATAGAT	7671–7649
Mu102F	GGCAACAACATTCACATTATATAGGA	5608–5633
Mu102R	GTAACCAATCCTATATAATGTGAATGTTGTTGCCTC	5641–5606
Mu189F	CCAAGTACAGTTGGAGGAGAACAGCAGTAAC	5867–5896
Mu189R	GTTATTGCTGTTCTCCTCCAACTGTAC	5897–5871
Mu192F	GGAGGAGAACAGCAGTAACATTATAA	5879–5904
Mu192R	TTATTCATTATAATGTTACTGCTGTTCTTCTCCAACTG	5911–5874
Mu235F	GTAGAAATGCAGCAAAGCGATAATAACACTTGGATTC	6003–6039
Mu235R	GAATCCAAGTGTTATTATCGCTTTGCTGCATTTCTAC	6039–6003
Mu504F	CAATAGCATGGAGTTAATAGAAGAGCAAATTCAT	6809–6842
Mu504R	TATGAATTTGCTCTTCTATTAACTCCATGC	6814–6843

The primers were located on the EIAV vaccine strain FDDV complete sequence. The underlined capitals indicate mutated nucleotides

cells about 4 days postinoculation. Aliquots of cell culture supernatant collected after the appearance of CPE were positive for reverse transcriptase activity (data not shown), which indicated that the two molecular clones, mFD5-4-7 and

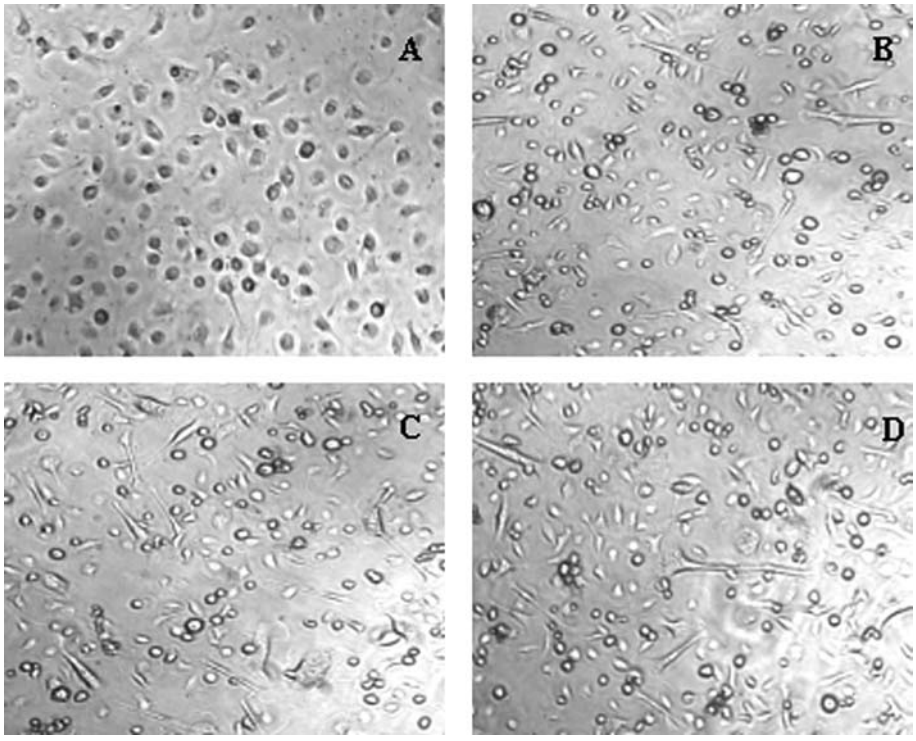


Fig. 3. Cytopathic effect (CPE) of three infectious molecular clones on DL cells. CPE appeared about 4 days postinfection. **A**: normal DL cells; **B**: pLGFD3; **C**: mFD5-4-7; **D**: mFD7-2-11

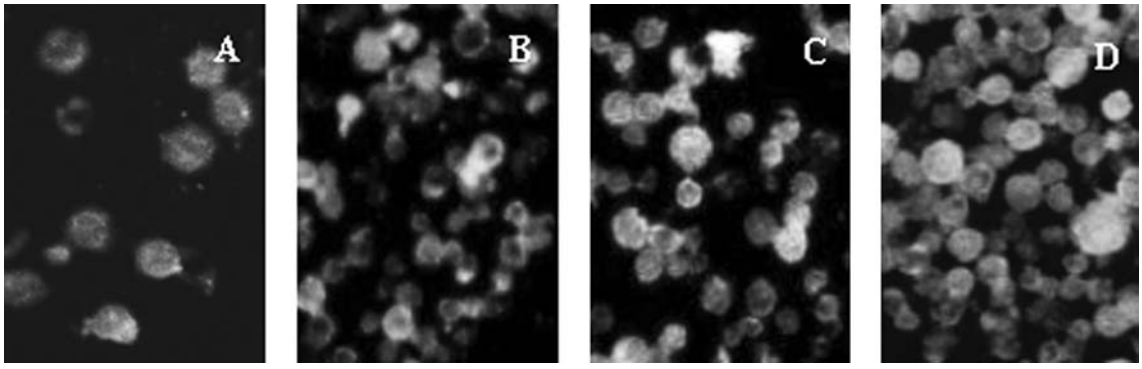


Fig. 4. Detection of protein expression of EIAV by immunofluorescence assay (IFA). IFA was performed using EIAV-positive serum and FITC-labeled rabbit antihorse IgG and counterstaining with 0.01% Evans blue. Positive signals were observed in all cells that were infected by the three infectious molecular clones. **A**: negative control (uninfected cells); **B**: pLGFD3; **C**: mFD5-4-7; **D**: mFD7-2-11

mFD7-2-11, were infectious clones and caused virus replication in both FDD cells and DL cells (Fig. 3).

Indirect immunofluorescence assay

To monitor the appearance of specific viral protein, virus stocks were inoculated onto FDD cells. About 10 days later (after appearance of CPE), cells were digested and smeared. Expression of viral protein was detected by an indirect immunofluorescence assay (IFA) using rabbit antihorse IgG conjugated with fluorescein isothiocyanate (FITC) as described in Materials and Methods. Fluorescence staining appeared in cultures infected with all three viral stocks (pLGFD3, mFD5-4-7, and mFD7-2-11 strains) but not in the uninfected cultures, demonstrating the expression of specific EIAV protein (Fig. 4).

Mutated virion observation on DL cells by electron microscope

To further confirm the presence of replication of mFD5-4-7 and mFD7-2-11, virion formation in DL cells was examined by electron microscopy (EM). CPE appeared in DL cells about 4 days postinoculation, and cells were collected to prepare for electron microscopy observation. Typical viral particles were visible in the cytoplasm as well as in the intercellular space (Fig. 5). This demonstrated that all three molecular clones could establish productive infection in DL cells and shed infectious virus particles into cells and cell culture medium. These results, together with the CPE and IFA observations, revealed that the viral stocks of the three molecular clones were able to infect *in vitro* culture systems and produce integrated viral particles.

Clinical and virologic profiles of experimental horses

To assess the virulence and replication properties of two point-mutated clones, six horses were inoculated with 10^5 MID of virus stocks of pLGFD3 (horses 15 and

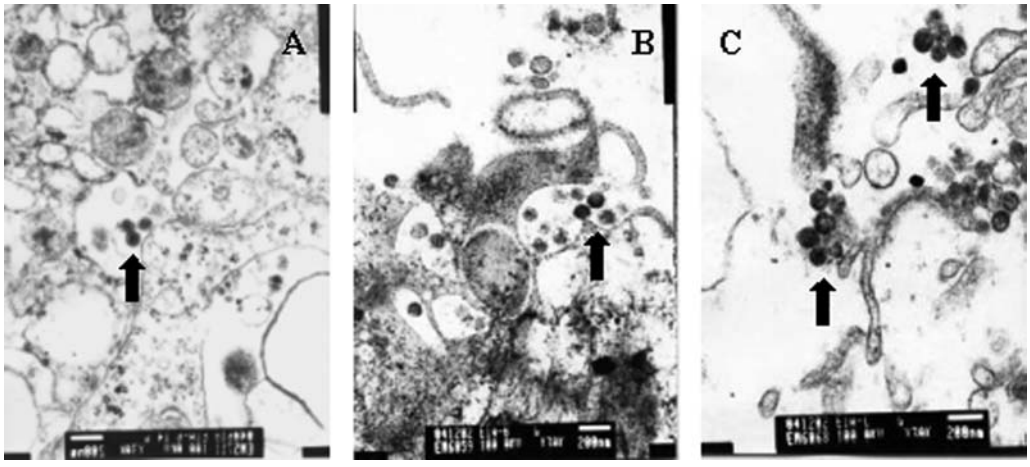


Fig. 5. Observation of virus particles of mutant molecular clones mFD5-4-7 and mFD7-2-11 and their parental clone pLGFD3 by electron microscopy (EM). Cells were collected when a cytopathic effect (CPE) appeared in DL cells. Typical viral particles in cytoplasm and intercellular space are indicated by arrows. **A**: pLGFD3; **B**: mFD5-4-7; **C**: mFD7-2-11

17), mFD5-4-7 (horses 32 and 33), and mFD7-2-11 (horses 34 and 39). The inoculated horses were monitored daily for clinical symptoms of EIA (fever, petechiae, etc.). Blood samples and plasma were collected for measurement of platelets and plasma viral load at regular intervals and during each febrile episode for up to 180 days as described in Materials and Methods. Clinical EIA episodes were defined when fever (rectal temperature ≧39 °C) occurred with thrombocytopenia (platelets $<100{,}000/\mu$l whole blood) and detectable plasma viral RNA.

After inoculation with pLGFD-3 strains, horses 15 and 17 remained asymptomatic for EIA as demonstrated by a lack of fever episodes and thrombocytopenia, sensitive indicators of EIA (Fig. 6) [22, 44]. This lack of detectable virulence by inoculation with the vaccine clone pLGFD-3 was associated with a relatively low level of plasma viral load, ranging from undetectable to 10^2 copies per ml plasma. The maintenance of low-level viral replication may be helpful in driving the maturation of immune responses to establish enduring and broadly protective immunity.

In contrast to the asymptomatic animals experimentally infected with pLGFD3, horses that were infected by either mFD5-4-7 or mFD7-2-11 strains developed typical EIA episodes. All clinical EIA episodes were associated with fever, thrombocytopenia, and plasma viral RNA levels in excess of 10^2 copies per ml of plasma. Horse 32 experienced one febrile episode at day 99 postinfection. Horses 33 and 34 both experienced two febrile episodes, at days 81 and 114 postinfection for horse 33 and at days 25 and 111 postinfection for horse 34. Horse 39 experienced multiple febrile episodes at days 6, 41, 67, and 89 postinfection (Fig. 6). The development of recurring EIA febrile episodes was characteristic of chronic EIA. The levels of virus replication also differed from those of horses inoculated with vaccine pLGFD3. Steady-state levels of plasma viral genomic

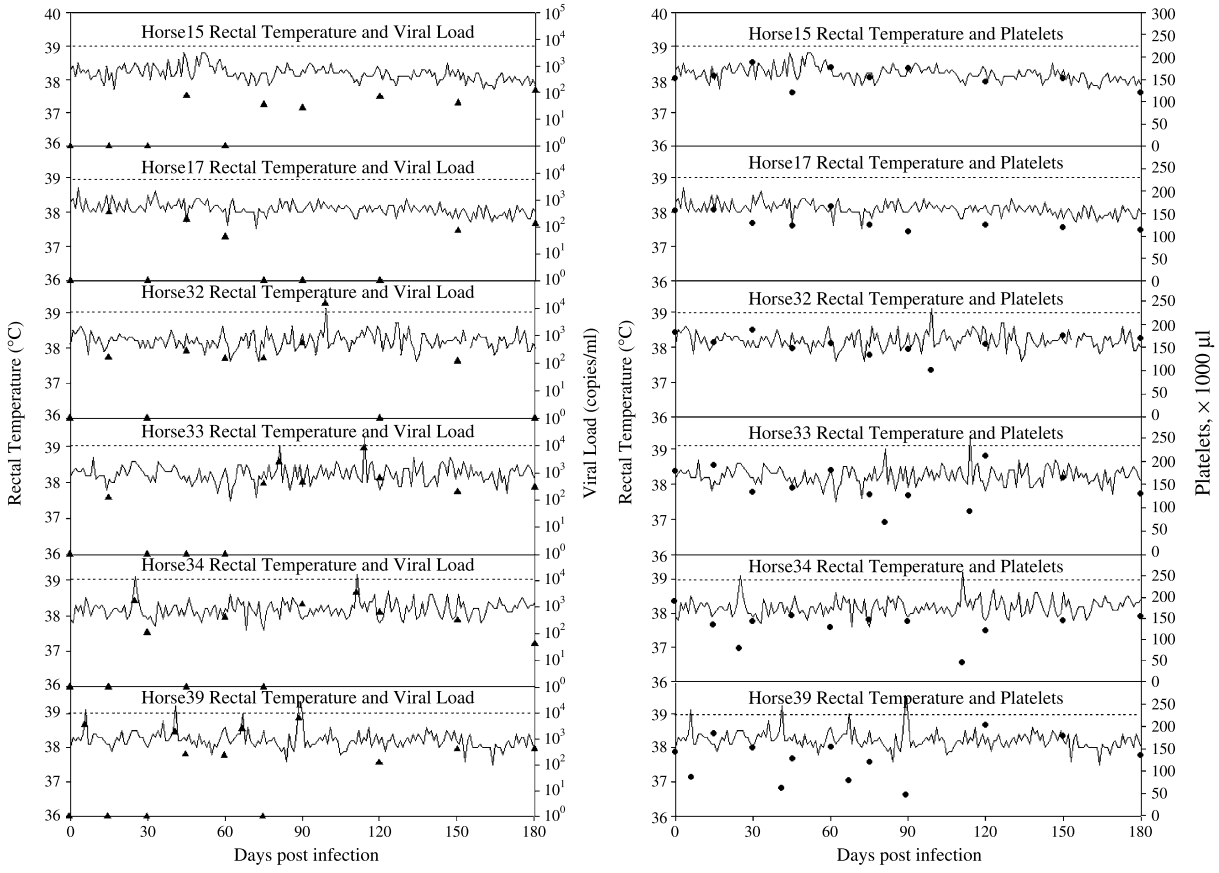


Fig. 6. Clinical disease progression and plasma viral load in experimental horses. Six horses were inoculated with EIAV infectious molecular clones as described in Materials and Methods. Rectal temperature (solid line, left y axis) was monitored daily. Platelet counts (●) and viral RNA copies (▲) in plasma were detected at regular intervals and during each febrile episode. Febrile episodes were defined as rectal temperature above 39 °C with thrombocytopenia (<100,000 platelets/µl whole blood) and detectable plasma viral load. The symbol ▲ on the x axis indicates an undetectable viral load

RNA in horses inoculated with mFD5-4-7 and mFD7-2-11 ranged from 10^2 to 10^4 copies per ml, much higher than those of horse 15 and 17 (less than 10^2 copies per ml). Thus, the two point-mutated clones mFD5-4-7 and mFD7-2-11 were both pathogenic and more febrile episodes were observed with mFD7-2-11 than with mFD5-4-7.

Discussion

During virus infection, envelope protein plays pivotal roles in the binding of receptors on target cells and then entering cells, inducing immune response and causing pathology. Based on amino acid sequence comparisons between Chinese pathogenic and vaccine strains of EIAV, critical consensus mutations were identified, with most of them located in the *env* gene of the viral genome, especially in

the gp90 glycoprotein (Fig. 1). Similar differences have been observed between the avirulent cell-culture-adapted $EIAV_{PR}$ strain and the virulent $EIAV_{PV}$ strain [13, 14], indicating that the majority of differences between the virulent and avirulent EIAV were primarily in the *env* region [32, 35, 39, 50, 52, 53]. This suggested that the region with the most variable sequences between EIAV virulent strains and avirulent or vaccine strains should possess some, if not all, of the pathogenic and immunological determinants. This prediction has been partially confirmed by the results of this study.

In this report, two mutant infectious clones, mFD5-4-7 and mFD7-2-11, were used to investigate virus produced and pathogenic properties *in vitro* and *in vivo*. Horses 15 and 17, inoculated with the pLGFD3 strain, did not experience any febrile episodes, remained asymptomatic, and maintained very low levels of plasma viral RNA (undetectable to 10^2 copies per ml) during the entire observation period. However, significant symptoms were observed in horses infected with mutant molecular clone strains. After infection, the first febrile episode appeared 6 days post infection in horse 39 and 25 days post infection in horse 34, both of which were inoculated with the mFD7-2-11 strain (6 point mutations), whereas horses 32 and 33, inoculated with the mFD5-4-7 strain (4 point mutations), did not experience a febrile episode until 99 and 81 days post infection, respectively. The delayed onset of disease (defined as clinical signs occurring after 42 days post infection) demonstrated the lower virulence of the mFD5-4-7 virus strain. Compared to pLGFD3, both mFD5-4-7 and mFD7-2-11 were pathogenic molecular clones with the appearance of recurring disease cycles. As we reported previously, however, horses infected with Chinese EIAV virulent strain LN40 usually experienced multiple and durative febrile episodes with accompanying viremia levels of over 10^8 copies per ml plasma [34]. Therefore, the two mutated clones mFD5-4-7 and mFD7-2-11, causing transient fever and viremia ranging from 10^2 to 10^4 copies per ml plasma, were weakly pathogenic compared with the virulent strain LN40. Horses infected with the mFD5-4-7 strain experienced even fewer febrile episodes than with mFD7-2-11, indicating that additional wild-type mutations caused additional pathology (Fig. 6), which is consistent with our previous results [23].

The envelope protein of lentivirus is extensively glycosylated [5, 10, 32]. One of the most important roles played by N-linked glycosylation associated with the *env* protein of HIV-1 and SIV is to mask the major neutralizing epitopes and allow variant viruses to escape recognition of host neutralizing antibody [5, 30, 42]. Also, it has been demonstrated that N-linked glycosylation facilitates the interactions of HIV receptor CCR5 with CD4 and assists in lentivirus infection [36]. We analyzed the potential N-glycosylation sites displayed in the *env* region and demonstrated that the virulent LN40 and D510 strains contain 25 and 22 potential N-glycosylation sites, respectively, while the attenuated vaccine DLV and FDDV strains possess 17 and 19 potential N-glycosylation sites, respectively (Fig. 1). The decrease in N-glycosylation sites in DLV and FDDV was attributed to mutations appearing at the N- or T/S-sites of the N-glycosylation motifs. Vaccine strains DLV and FDDV have fewer N-glycosylation sites, which may expose more neutralizing

epitopes, leading to a longer and stronger immune response [12]. In this study, mutation of residues 192 (Ser → Asn), 235 (Asp → /) and 236 (Asn → Lys) in *env* resulted in the loss of two N-glycosylation sites (Fig. 1). Mutations of 189 and 192 were located in the putative principal neutralizing domain (PND) and mutations of 235 and 236 were located downstream of PND. Removing the above two N-glycosylation sites from the virulent EIAV gp90 protein may structurally and functionally affect the predominant T- and/or B-cell epitopes that were distributed in the corresponding region and change the sensitivity of the virus to neutralizing antibody and CTL response [12, 19, 27]. These results suggest that the N-linked glycosylation in the SU protein of EIAV could conceal antigenic epitopes from the immune system and confer some resistance to neutralizing antibodies and CTL response.

Of ten mutations in the *env* region, only one (residue 505, Val/Ile → Thr) occurred in gp45. The amino acid at position 505 is located in the third N helix of gp45, which is responsible for viral fusion activity [9, 18]. Previous data showed that point mutations in the N helix of gp41 can influence its fusion activity and infectivity [29]. *Env*-mediated fusion relies on the formation of a six-bundle helix, internal triple-stranded N-peptide helices paired with antiparallel outer C-peptide helices. When amino acid 36 in the N helix of gp41 was mutated from Gly to Asp, the fusogenic activity was dramatically decreased. This was ascribed to the physical impediments of the six-helix bundle formation since the protrusion of the negatively charged 36D residue in the N helix would induce a steric clash [29]. Gp45 has three N helices and three C helices in its ectodomain, and residue 505 is located within the third N helix [9, 18]. Mutation of amino acid 505 from Val/Ile to Thr converts a hydrophobic amino acid to a hydrophilic one and shortens the side chain of the amino acid slightly. In our study, viral stocks of clone mFD7-2-11 with 505Thr, demonstrated more febrile episodes than pLGFD3 and mFD5-4-7 with 505Val (Fig. 6). These changes were very similar to those of the gp41 mentioned above, an inefficient membrane fusion with mutation of residue 36 Gly → Asp. It could be proposed that amino acid residue 505 of EIAV gp45 is also a critical regulator for the structural and functional changes of the ectodomain, influencing the infectivity and pathogenicity through fusion activity changes just like that which occurred in gp41.

Despite the pandemic of HIV, a safe and effective vaccine to control HIV-1 remains unavailable. One approach focuses on live attenuated virus vaccines, which typically have the advantage of inducing a broad and long-lived immune response. The SIV/macaque model is generally accepted for AIDS vaccine studies and has been widely used to evaluate the safety and efficacy of candidate AIDS vaccine. The live attenuated SIV vaccine was developed by deleting the *nef* or *nef* plus *vpr* genes [15, 20]. However, recent studies found that persistent infection and pathogenicity occurred after vaccination of rhesus macaques with SIVmac239Δ3, a *nef*-deleted vaccine strain that had been further attenuated by additional large deletions in *vpr*, the long terminal repeat that overlaps with the remaining *nef* coding region and the negative regulatory element [20]. Most monkeys vaccinated as infants progressed to AIDS [2, 25], with the majority of adult

monkeys developing chronic recurrent viremia during long-term follow-up of up to 6.8 years.

It has been shown that the *env* gene of EIAV is related to viral virulence [3, 37, 41]. In this study, our finding further demonstrates that several critical amino acid sites of *env* gene are clearly associated with virulence. The dramatic differences in clinical progression and plasma viral load of experimental animals were ascribed to the combined mutations involving 6 critical residues in the *env* region. These results connect viral gene variation with its biological characteristics, which may help elucidate the attenuation mechanism of EIAV and provide an instructive theoretical basis for the development of a potential HIV vaccine. Future investigation of site-specific mutagenesis of these consensus critical mutation sites from virulent EIAV strains to vaccine strains will further confirm the influence of variation in these relatively few nucleotides on viral virulence and pathogenesis of lentivirus.

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