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Amino acid mutations of the infectious clone from Chinese EIAV attenuated vaccine resulted in reversion of virulence

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Abstract

The Chinese equine infectious anemia virus (EIAV) donkey-leukocyte attenuated vaccine (DLV) provides a unique natural model system by which attenuated mechanism and immunological control of lentivirus replication may be studied. We analyzed the critical consensus mutations that occurred during the viral passages in vitro and in vivo for vaccine's preparation. Based on the full-length infectious clone pLGFD3 (EIAV vaccine background) and according to mutations displayed during viral attenuation, we successfully constructed an infectious clones pLG5-3-l in which *gag* and *env* genes were point-mutated by overlap PCR mutagenesis strategy. pLG5-3-l was proved to have the ability of effective replication in vitro cells culture systems by Reverse Transcriptase Assay and virion observation under electron microscopy. Results of the in vivo experiments indicated that marked differences occurred between the mutated virus and their parental virus in clinical manifestation and plasma viral replication during 6-month observation period. In contrast to asymptom of animals infected with pLGFD3-V, the mutated virus (pLG5-3-l-V) developed typical clinical progression in the corresponding experimentally infected animals. The results of the distinct differences in clinical profiles and viral dynamics before and after mutation of EIAV infectious clone will help to understand the protective mechanism of Chinese EIAV vaccine and shed light on novel HIV vaccine design.

Keywords: EIAV; Attenuated vaccine; Mutated infectious clone

1. Introduction

Both equine infectious anemia virus (EIAV) and HIV belong to members of the lentivirus subfamily of retroviruses. Though the clinical manifestations of infections by EIAV and HIV are quite different, the underlying mechanisms of persistence and pathogenesis are very similar [1,2]. These similarities are reflected in common genetic structure, molecular mechanism of viral replication and 3D structures of main structure proteins [3–6]. EIAV is a macrophage-tropic lentivirus that can cause an acute, chronic and asymptomatic infection in horses according to different levels of viral replication in peripheral blood and tissue macrophages and host immune status [7,8]. Most of chronic infected horses survive inapparent carrier phase after recurring cycles of fever, anemia, weight loss, and thrombocytopenia [8,9]. Virus-specific immune responses are considered as the critical factors of switching from disease phase to asymptomatic infection, which mainly involve the emergence of EIAV-specific CD8+ cytotoxic T lymphocytes and humoral immune responses, including neutralizing and non-neutralizing antibodies [10,11].

The Chinese EIAV donkey-leukocyte attenuated vaccine (DLV) was developed in 1978 [12,13]. The generation of Chinese EIAV attenuated vaccine included three stages. Firstly,

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a carefully selected field isolate was passaged in horses for 16 generations to obtain a pathogenic strain, named LN40 (GenBank accession no. AF327877); secondly, LN40 was then passaged 133 times in donkeys resulting to gain a more virulent strain D510 which yielded 100% acute EIA symptom and more than 90% mortality in experimentally infected horses in the first 50 days after inoculation; finally, the DLV (GenBank accession no. AF327878) was developed from D510 through 120 successive passages in vitro in primary donkey macrophages [13]. Thereafter, by the same method, another fetal donkey dermal cells adapted vaccine (FDDV) was developed by passaging DLV in fetal donkey dermal (FDD) cells for 15 times and FDDV was proved to have more excellent effect of immune protection than that of DLV [14]. The vaccine strains have been proved to be stable, and no virulent reversion was detected in over 500 horses and donkeys inoculated with DLV [13]. Also, no significant local or systemic side effect was observed. DLV vaccinated animals could resist challenge from homogeneous and heterologous virulent EIAV from China as well as North America [13].

In order to unveil the genetic basis of the success with these attenuated vaccines, we first selected EIAV *gag* and *env* region as research targets because studies on the important determinants of pathogenesis of North American EIAV strains indicated that the *gag* and *env* region might contribute to virulence [15–17]. It was also reported that EIAV infected animals developed a strong immune response against the surface (gp90) and transmembrane (gp45) glycoproteins and the major core protein (p26) [10,18,19]. However, there is no such report about Chinese EIAV strains in spite of the significant genetic differences between Chinese EIAV and North American EIAV strains. EIAV envelope includes a defined principal neutralizing domain (PND) [20] and a variety of N-linked glycosylation sites [21,22], which are possibly responsible for weakening host immune defenses. Multiple broadly recognized T-cell epitopes are also demonstrated to distribute within *gag* region [19].

In this paper, we constructed an EIAV full-length infectious molecular clone based on plasmid pLGFD3 (a full-length infectious clone of FDDV) in which most of the defined critical consensus mutation sites displayed within *gag* and *env* region (3 amino acids in *gag* and 4 amino acids in *env*) were mutated. Biological properties of the mutated clone and its parental clone were compared in vivo. Virus stock of the mutated clone was inoculated into two experimental horses and both of them presented typical clinical manifestation of EIA. The results demonstrated that virus stock of mutated clone had similar characteristics with EIAV pathogenic strain in term of infectivity and replication activities, which indicated that the combined point-mutations of *gag* and *env* gene could reverse the nonpathogenic clone to a pathogenic one. This reversion may due to the combined effect of mutations of several amino acids sites. All of these results provided a basis for further study on the pathogenic mechanisms of EIAV and the immune protection of Chinese EIAV vaccines.

2. Materials and methods

2.1. Computer-assisted sequence analysis

Clustal and *GCG* software were used for cluster analysis of protein primary structure and the results of alignments were edited by *Genedoc* software.

2.2. Plasmid, virus strain, and cell culture

Fetal donkey dermal cells and donkey primary lymphocytes (DL) culture systems were provided by Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences. FDD cell cultures were described as below and DL cell cultures were prepared from heparinized peripheral blood as equine MDM (monocyte/macrophage differentiation-maturation system) cells [23]. Briefly, supernatant plasma (contain mononuclear cells) was isolated from fresh heparinized donkey whole blood after stayed in 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 (Sigma)- and plasma-coated tissue culture dish (150 cm^2) (Corning) 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 wilh 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/well.

The avirulent full-length infectious molecular clone pLGFD3 was constructed based on the Chinese EIAV fetal donkey dermal cells adapted vaccine (FDDV) [24]. Virus derived from the molecular clone (pLGFD3-V) was generated by transfecting purified pLGFD3 DNA into FDD cells with DOTAP Liposomal Transfection System (Roche) according to the manufacturer's instructions and then sequentially passaged in FDD cells for 9 generations and further passaged in DL cells for 4 generations. The mutated full-length molecular clone pLG5-3-l (its derived virus was designated as pLG5-3-l-V) was described below.

2.3. Experimental subjects, clinical evaluation, and sample collection

All the horses (15#, 17#, 23#, 24#, 31#) used in these studies were thoroughbreds. Prior to the experiment, serum samples from all horses were tested twice by the agar gel immunodiffusion test (VMRD, Inc.) to ensure that they were seronegative for EIAV [25]. They were housed in screened box stalls to exclude hematophagous insects, and all animal handling protocols were confirmed by Animal Management Committee of Chinese Harbin Veterinary Research Institute. Rectal temperature and clinical status were recorded