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Peripheral immunophenotype and viral promoter variants during the asymptomatic phase of feline immunodeficiency virus infection

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ABSTRACT

Feline immunodeficiency virus (FIV)-infected cats enter a clinically asymptomatic phase during chronic infection. Despite the lack of overt clinical disease, the asymptomatic phase is characterized by persistent immunologic impairment. In the peripheral blood obtained from cats experimentally infected with FIV-C for approximately 5 years, we identified a persistent inversion of the CD4/CD8 ratio. We cloned and sequenced the FIV-C long terminal repeat containing the viral promoter from cells infected with the inoculating virus and from *in vivo*-derived peripheral blood mononuclear cells and CD4 T cells isolated at multiple time points throughout the asymptomatic phase. Relative to the inoculating virus, viral sequences amplified from cells isolated from all of the infected animals demonstrated multiple single nucleotide mutations and a short deletion within the viral U3, R and U5 regions. A transcriptionally inactivating proviral mutation in the U3 promoter AP-1 site was identified at multiple time points from all of the infected animals but not within cell-associated viral RNA. In contrast, no mutations were identified within the sequence of the viral *dUTPase* gene amplified from PBMC isolated at approximately 5 years post-infection relative to the inoculating sequence. The possible implications of these mutations to viral pathogenesis are discussed.

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1. Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that infects cats, resulting in an acute infection syndrome followed by a prolonged asymptomatic period during which the CD4/CD8 T cell ratio is inverted (Ackley et al., 1990; Barlough et al., 1991; Joshi et al., 2004; Kohmoto et al., 1998; Pedersen and Barlough, 1991; Torten et al., 1991). FIV causes progressive immunologic impairment, culminating in an AIDS-like syndrome and death, akin to HIV-infected humans (Ikeda et al., 1996; Joshi et al., 2004; Kohmoto et al., 1998). The FIV-infected cat is the only naturally-occurring, outbred, large animal model of lentivirus-induced immunodeficiency and like HIV, FIV is capable of infecting both CD4 T cells and monocytes in the susceptible host (Bendinelli et al., 1995; Burkhard and Dean, 2003; Joshi et al., 2004).

Our laboratory has established a model of lentiviral cellular latency in experimentally FIV-infected specific pathogen free (SPF) cats during the asymptomatic phase of infection (Murphy et al., 2012). SPF cats infected with a biological isolate of FIV clade C (Pgmr) for approximately 3 years have an estimated proviral load of 1 infected peripheral CD4 T cell in approximately 10^3 peripheral CD4 T cells (McDonnel et al., 2012b). Our laboratory has experimentally determined that of those infected CD4 T cells, there is approximately 1 copy of viral DNA per cell and 1 in 10 proviral copies appear capable of transcription after *ex vivo* activation. In latently infected peripheral CD4 T cells, the integrated and transcriptionally inactive FIV promoter is physically associated with deacetylated, methylated histone proteins, consistent with a restrictive chromatin environment (McDonnel et al., 2012b). The latent provirus can readily be reactivated *in vitro* with exposure to histone deacetylase inhibitors such as suberoylanilide hydroxamic acid (SAHA), which result in histone acetylation at the integration site of the proviral promoter and transcriptional activation of the provirus (McDonnel et al., 2012a).

A single nucleotide mutation within the FIV-C U3 AP-1 site was previously shown to abrogate transcription in a β galactosidase reporter gene assay (Murphy et al., 2012). This AP-1 mutation was found to be present in the proviral DNA of CD4 T cells isolated from all of the FIV-infected cats. Lentiviral latency has been defined as a reversible low-productive state of infection, where infected cells retain the capacity to produce new viral particles (Eisele and Siliciano, 2012). Although we have previously demonstrated that latency is associated with a restrictive chromatin environment, we wondered whether the AP-1 mutation might be associated with an additional mechanism of viral latency. Although our *in vitro*

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experiment indicated transcriptional abrogation, *in vivo* viral latency mechanisms may be more complex (*e.g.* AP-1 mutation associated with leaky or low-level viral transcription in certain cellular states). We hypothesized that the FIV-C proviral U3 AP-1 mutation is associated with intermittent/low-level viral transcription and therefore, latency.

For the study reported here, serial peripheral blood samples were obtained from FIV-infected cats and mock-infected control cats throughout the asymptomatic phase and were systematically analyzed for detectable plasma virus and the enumeration of total white blood cells and cellular subsets using surface antigen-specific antibodies (anti-CD4, CD8, MHC II, CD11b, CD21 and CD25). Nucleic acids isolated from peripheral blood mononuclear cells (PBMC) and peripheral CD4 T cells were analyzed for detectable viral promoters *via* nested PCR; amplified viral promoters were subsequently cloned and sequenced. Since lentiviral latency is likely mechanistically attributable to the host/viral promoter interface, we focused our sequencing efforts on the viral promoter. During the study, multiple G to A transition mutations were identified in the proviral LTR. Since it has previously been demonstrated that FIV lacking a functional *dUTPase* gene is prone to G to A transition mutations, the FIV-C *dUTPase* gene was also amplified and sequenced.

2. Materials and methods

2.1. Animals

Six FIV SPF kittens were purchased from the breeding colony of the Feline Nutrition and Pet Care Center, University of California at Davis (UC Davis). At time of purchase, the kittens ranged in age from 4 to 5 months and were housed in the Feline Research Laboratory of the Center for Companion Animal Health, UC Davis. Four kittens were intramuscularly inoculated with FIV-C-Pgmr viral inoculums (kittens 165, 184, 187 and 186) and monitored as described previously (Murphy et al., 2012). Two control kittens (183 and 185) were mock-inoculated with 1 ml of sterile culture media. The FIV-C-Pgmr biological isolate was provided by Drs. E. Hoover (Colorado State University) and N. Pedersen (University California, Davis). This study spans the time of inoculation to approximately 253 weeks post-infection (5 years). Blood samples were obtained approximately once a month throughout this time period. The study protocol was approved by the UC Davis Institutional Animal Care and Use Committee.

2.2. Plasma virus

Whole blood was collected from FIV-infected and uninfected cats every 2–4 weeks *via* jugular venipuncture in EDTA-containing tubes and centrifuged at 500 × *g* for 5 min. Plasma was subsequently transferred and centrifuged at 17,000 × *g* for five additional minutes. Viral RNA was isolated from clarified plasma using a commercially available kit (QIAmp Viral RNA Minikit, Qiagen). Isolated vRNA was DNase treated (Turbo DNase, Ambion) and reverse transcribed using the First-Strand cDNA Synthesis System for Quantitative RT-PCR (OriGene). A control reaction excluding reverse transcriptase was included for each set of reverse transcribed cDNA.

Isolated RNA was then assayed for the presence of FIV *gag via* real-time PCR utilizing the primers $FIV_{QT\ gag\ for}$ and $FIV_{QT\ gag\ rev}$, as described previously (Murphy et al., 2012). Real-time PCR was performed in triplicate with Real Mastermix SYBR Rox (5 PRIME) on an Applied Biosystems 7300 Real-Time PCR System with the following cycling conditions: 50 °C for 2 min, 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s, 68 °C for 30 s and a final elongation step at 72 °C for 5 min as previously described (Murphy et al., 2012). The real-time PCR assay has a detection limit of approximately 10 copies of FIV *gag* (data not shown). All real-time PCR assays were followed with a dissociation step (melt curve) to assess amplicon validity.

2.3. Leukocytes

The total WBC concentration was determined from whole blood collected in EDTA-containing tubes with a commercially available system (LeukoChek, Biomedical Polymers, Inc.) coupled with hemocytometer enumeration. The relative proportion of specific leukocyte subsets was assessed utilizing the following antigen-specific antibodies: anti-feline CD4 (clone FE1.7B12), anti-feline CD8 (clone FE1.10E9), anti-canine CD21 (clone CA2.1D6), anti-canine 11b (clone CA16.3E10), anti-feline MHC II (clone 42.3), and anti-feline CD25 (clone 9F23). All of the antibodies were obtained from Dr. Peter Moore (UC Davis) with the exception of anti-feline CD25, which was a gift of Koichi Ohno, (University of Tokyo). The proportion of cells expressing a specific marker was determined by flow cytometry, using a procedure described previously (Murphy et al., 2012). Briefly, 100 μl of whole blood was incubated at room temperature with each of the antibodies listed above. The cells were subsequently incubated with eythrocyte lysis buffer (154 mM ammonium chloride, 82 mM potassium bicarbonate and 18 mM EDTA tetrasodium salt in phosphate buffered saline (PBS), pH 7.2), washed with flow buffer (2 mM EDTA trisodium salt, 2.55 mM ETA disodium salt and 15 mM sodium azide in PBS, pH 7.2), stained with a secondary fluoroscein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (Vector Laboratories, Inc.) and incubated in the dark for 15 min. Cells were washed a final time with flow buffer and centrifuged at 500 × *g* for 5 min. Pelleted cells were resuspended in flow buffer and analyzed using a FACScan flow cytometer (Becton Dickinson); resulting data was analyzed with FlowJo v8.6.3 (Treestar). CD4 and CD8 cell counts were derived from the absolute lymphocyte counts (multiplying the absolute lymphocyte number by the percent of cells expressing the CD4 or CD8 marker); the CD4/CD8 ratio was determined by division of the percent expression of the two markers. Viable peripheral CD4 T cells were purified from whole blood as described previously using feline antigen-specific antibodies and magnetic columns (Murphy et al., 2012).

2.4. FIV LTR amplification, cloning and sequencing

The proviral LTR was amplified from nucleic acid isolated from cryogenically stored preparations of single passage feline PBMC infected with the inoculating virus along with FIV-infected cat-derived peripheral blood mononuclear cells (PBMC) and column-isolated CD4 T cells obtained throughout the infection. PBMC were isolated from peripheral blood using Ficoll-Hypaque (Sigma), DNA was extracted (DNA Mini Kit, Qiagen), the proviral LTR was amplified *via* nested PCR (described below) and cloned using a commercial system (pCR2.1, TA cloning system, Invitrogen). Plasmid DNA was purified *via* a commercial kit (Promega) and the inserted DNA was sequenced by a local vendor (Davis Sequencing). Nucleotide sequences were aligned and compared using the *AlignX* function of Vector NTI software (Invitrogen).

Freshly column-isolated CD4 T cells were co-cultured at a ratio of 1:1 with allogeneic SPF feline PBMC at a final concentration of 1×10^6 cells/ml for 7–23 days using previously described protocols (Sparger et al., 1994) with the addition of mitogens (phorbol myristate acetate, 0.5 μg/ml, Sigma–Aldrich) and Concanavalin A (5 μg/ml, ThermoFisher Scientific). Cell-associated DNA and RNA were co-isolated (AllPrep DNA/RNA Mini kit, Qiagen) at incubation days 0, 7 and 23. RNA was DNase treated (TURBO DNase, Ambion)

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