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Viral Gene Expression and Provirus load of Orf-A defective FIV in Lymphoid Tissues and Lymphocyte subpopulations of Neonatal Cats During Acute and Chronic infections

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Abstract

Neonatal cats were infected with a wild type (JSY3) or orf-A defective (JSY3ΔORF-A) feline immunodeficiency virus (FIV) to determine the provirus load and level of viral gene expression at the acute versus chronic stages of infection. FIV DNA in the thymus, lymph node, peripheral blood mononuclear cells (PBMCs) and lymphocyte subpopulations at week 8 post-infection was lower in animals infected with JSY3ΔORF-A as compared to that of JSY3. At week 16 we observed no significant difference in provirus load between the two groups except for B cells where it was higher in the JSY3 infection. In B cells proviral burden was found to be the same in animals infected with JSY3 for both time points. In the chronic stage, therefore, proviral burden dominates in B cells for JSY3, whereas the level of JSY3ΔORF-A was lower with comparable values for all lymphocytes at both weeks 8 and 16. Gene expression profiles as measured by real time PCR for gag and rev transcripts revealed decreased levels of JSY3ΔORF-A mRNAs as compared to that of JSY3. The JSY3 chronic phase infection showed viral gene expression to be higher in B cells relative to CD4+ and CD8+ cells. The presence of viral RNA in CD8 and B cells during the chronic infection implicates active virus replication. Hematological profiles revealed that there was a decline in the number of B cells in JSY3ΔORF-A-infected cats during the chronic stage of infection while no significant change was observed in animals infected with the wild type virus. Comparative analysis of cell numbers to provirus load and levels of viral transcripts in CD4+ and CD8+, however, did not correlate cell numbers to the levels of viral DNA and gene expression. It remains to be determined whether the relatively high virus burden in B cells as compared to CD4+ and CD8+ cells reflects a role for Orf-A in a shift to B cell virus load during the chronic stage of FIV infection.

Keywords

FIV; Orf-A; Cats; thymus; lymph node; PBMCs; lymphocytes

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1. Introduction

Feline immunodeficiency virus (FIV), a lentivirus of cats, causes an acquired immunodeficiency syndrome (AIDS) similar to that which is seen in humans with the human immunodeficiency virus (HIV). The clinical course of FIV infection parallels what is seen in HIV and therefore provides a small animal model. FIV is characterized by an initial acute infection (fever, malaise, neutropenia) with a resulting clinically latent infection (low plasma viremia, lymphadenopathy) where the subject displays no overt clinical illness (Ackley et al., 1990; Davidson-MG et al., 1993; McChesney and Oldstone, 1989; Pedersen, 1989; Pedersen et al., 1987; Willett et al., 1997a; Yamamoto et al., 1989). During the course of infection, CD4⁺ T cells are infected and depleted resulting in an inverted CD4⁺/CD8⁺ T cell ratio causing immunosuppression and a gradual wasting disease. Although FIV and HIV affect the CD4⁺ T cell population, data show that they display different cellular tropisms (Dean et al., 1999; Phillips et al., 1990; Rigby et al., 1997; Sparger et al., 1994; Sparger et al., 1997; Willett et al., 1997b). FIV has a broader host range and infects CD4⁺, CD8⁺, B cells and macrophages. HIV-1 infects cells by binding to its primary receptor the CD4 surface molecule and by interacting with chemokine receptors, mainly CXCR4 and CCR5 (Berger et al., 1999; Sattentau and Weiss, 1988). The primary receptor for FIV was recently identified to be the CD134 molecule which was found on the surface of several cell types (De Parseval et al., 2004; Shimojima et al., 2004). CXCR4 serves as a co-receptor for FIV infection (Willett et al., 1997b).

Similar to HIV, the FIV genome contains several open reading frames (ORFs) in addition to genes encoding for structural proteins (Olmsted et al., 1989; Talbot et al., 1989). These additional ORFs include the *vif*, *rev* and *orf A* genes. A mutational analysis of the *vif* gene reveals that this auxiliary gene product is important for efficient viral replication (Shacklett and Luciw, 1994). The FIV *rev* gene product is critical for virus replication (Kiyomasu et al., 1991; Phillips et al., 1992; Tomonaga et al., 1993). Furthermore, an unusual RNA-folding region, Rev response element (RRE), that is required for Rev has been identified at the end of *env* (Kiyomasu et al., 1991; Phillips et al., 1992). This region reveals a structure that is highly conserved among several FIV isolates (Phillips et al., 1992). FIV replication is dependent on a fully functional Rev protein and RRE for productive replication (Kiyomasu et al., 1991; Tomonaga et al., 1993). The *orf-A* gene product has remained elusive and its function in virus replication is not completely understood. Initially, Orf-A was demonstrated to have a minor transactivation (3-7 fold) activity on gene expression under the control of the FIV long terminal repeat (LTR) (De Parseval and Elder, 1999; Sparger et al., 1992). Recent reports implicate that the Orf-A may have multiple functions similar to the HIV accessory proteins. Gemeniano et al were able to demonstrate that Orf-A has a small effect on FIV gene expression and concluded that the protein is required for virus particle formation and virus infectivity (Gemeniano et al., 2003). Further studies by the same group have shown that a green fluorescent protein (GFP) fused with Orf-A gets localized to the nucleus and that Orf-A induces cell cycle arrest at the G2 phase similar to Vpr of HIV (Gemeniano et al., 2004). These findings suggest that Orf-A may serve as an adaptor molecule for several functions that help viral replication.

Infection studies with ORF-A defective FIV show that virus replication is reduced both in vitro and in vivo. The molecular clone, FIV-p34 is a natural ORF-A defective clone (Talbot et al., 1989). Sparger et al demonstrated that FIV-p34 showed reduced pathogenicity and low viral load in cats (Sparger et al., 1994). Over 50% of the cats studied failed to develop viral antibody, and were consistently virus positive only by PCR of proviral DNA. However, no comparative analysis was performed with FIV-p34 containing intact ORF-A, instead the study included infection with FIV-pPPR for comparison. Although all cats inoculated with cloned virus FIV-pPPR seroconverted, both clones were less capable of suppressing CD4⁺:CD8⁺ ratios when compared to the biological isolates. Inoshima and colleagues also investigated the role of ORF-

A during an in vivo FIV infection (Inoshima et al., 1996). Using an orf-A mutant of the TM2 molecular clone of FIV, they observed a delayed antibody response and only mild decreases in the CD4⁺:CD8⁺ ratios when compared to the wild type (WT) TM2 molecular clone. The only histological changes noted were the enlargement of germinal centers in the lymph node of two ORF-A mutant infected animals. Interestingly, the WT TM2 molecular clone also failed to cause histological change in the thymus in two of the three infected animals. Virus was detected by polymerase chain reaction (PCR) and immunohistochemistry in the lymph nodes of both the ORF-A mutant and WT TM2 infected animals. However, in the thymus provirus was only detectable by PCR in both mutant and WT TM2 infected animals. Only a single WT TM2 infected animal demonstrated virus by immunohistochemistry in the thymus.

Our earlier study in acutely infected cats with the highly pathogenic JSY3 clone and an ORF-A deficient mutant, JSY3ΔORF-A, showed a significant reduction of viral replication in the JSY3ΔORF-A infected cat (Norway et al., 2001). In both wild type and mutant virus infected cats, cell-associated viral load varied in a tissue-dependant manner with replication highest in lymphocytes isolated from the thymus, then the peripheral blood, and lowest in lymph node. Thymic proviral load and the number of viral p24 Gag positive cells were also reduced in cats infected with JSY3ΔORF-A. Furthermore, a reduction of the peripheral blood CD4:CD8 ratio was delayed in JSY3ΔORF-A infected cats. In addition, thymocyte subpopulations demonstrated a reduced expansion of single positive CD4-CD8⁺ thymocytes in JSY3ΔORF-A infected cats compared to animals infected with JSY3. The observation that there is a reduction in virus replication and a delay in the onset of the parameters that measure immunodeficiency in cats infected with an ORF-A defective mutant suggests that this protein is important in the progression of disease caused by FIV. To establish a better understanding of the role of Orf-A in virus replication and disease progression, we examined the influence of Orf-A in provirus load and viral gene expression in tissues as well as in different subpopulations of lymphocytes in an acute versus chronic FIV infection.

2. Materials and Methods

2.1 Cell Lines

A human fibroblast 293T cell line was cultured in complete DMEM, supplemented with 200nM L-Glutamine, 10% fetal bovine serum, 100 units/ml Penicillin and 100 ug/ml Streptomycin. 293T cells were incubated at 37°C in 5% CO₂. A feline CD4⁺ cell line, CD4E, was cultured in complete RPMI medium 1640 (cRPMI) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 0.075% sodium bicarbonate, 2 mM sodium pyruvate, 2-mecaptoethanol, and 100 units/mL recombinant human interleukin 2 (rIL-2, provided by the NIH AIDS Research and Reference Reagent Program, Rockville, MD). Cultures of CD4E cells were incubated at 37°C in 7% CO₂.

2.2 Plasmid constructions

FIV Provirus molecular clone JSY3 was provided by W. A. F. Tompkins (North Carolina State University, Raleigh). Cellular sequences flanking the FIV provirus were removed by PCR strategy and the Provirus was cloned into the restriction enzyme sites *Sph*I and *Acc*I of pSP72 (pSP72JSY3). Subsequently the entire U3 region was replaced with the cytomegalovirus immediate early gene promoter (CMV) and transcription initiates at the beginning R of the 5' LTR, creating pCMVJSY3. Similarly in an Orf-A defective FIV (JSY3ΔORF-A) the U3 region at the 5' end was also replaced by a CMV promoter generating pCMVJSY3ΔORF-A. The construction of JSY3ΔORF-A was described previously (Norway et al., 2001). In JSY3ΔORF-A the provirus contained two doublet nucleotide deletions (nt 6013/6014 and 6028/6029 of the JSY3 genome) of orf-A which allows premature termination of ORF-A at the amino-terminus.

2.3 Virus propagation

For virus preparation the human cell line, 293T, (plated at a concentration of 4×10^5 cells/mL) was transfected with 1.5 μ g of pCMVJSY3 or pCMVJSY3ORF-A/2 plasmid in six well plates by the liposome-mediated method with the reagent LipofectAMINE™ according to manufacturer's recommendations (Life Technologies, Inc., Gaithersburg, Md.). The 293T cells were grown in Dulbecco's modified Eagle medium supplemented with antibiotics and 10 % fetal calf serum. Twenty-four hours post transfection the medium was replaced with RPMI containing 10% fetal calf serum and 100ug/ml IL-2. A feline derived CD4+ cell line (CD4E), at 1×10^6 cells/ml, was co-cultured with FIV provirus transfected 293T cells. The CD4E cells were removed four days post co-culture and replated. Virus production was monitored at intervals by reverse transcriptase assay as described previously (Lee et al., 1987). Supernatants were harvested from time points with the optimum RT activity and used to infect fresh CD4E cells to prepare virus stocks. TCID₅₀ values were determined by serially diluting supernatants of each virus stock, infecting CD4E cells and counting syncytia formation as described previously (Bellah and Bubenik, 1998). DNA was extracted from titer cultures of pCMVFIVORF-A virus infected cells and the orf-A mutation was confirmed by sequencing double-stranded DNA templates using the Perkin-Elmer Applied Biosystem Division (PE/ABD) 373A automated sequencer.

2.4 Animals and inoculations

One-day-old kittens (n=16) were infected with virus from either the wild type clone JSY3 (n=6) or ORF-A mutant JSY3 Δ ORF-A (n=10) with a dose of 10^4 TCID₅₀. The animals were injected by the intraperitoneal (IP) route with a 200 μ l volume of virus inoculum. Kittens infected with JSY3 or JSY3 Δ ORF-A virus were randomly grouped for acute (8 weeks) or chronic (16 weeks) phase studies.

2.5 Analysis of hematological profiles

To determine the CD4:CD8 ratio of the cats infected with either JSY3 or ORF-A mutant FIV, biweekly hematological profiles were assessed as we have previously performed (Norway et al. 2001; Orandle et al., 2000). After inoculation, blood samples were collected biweekly for hematological assays and complete blood counts with differentials being determined by standard methods (Barlough et al., 1990). Peripheral blood lymphocyte profiles were analyzed by dual fluorescence flow cytometry in both groups of infected cats as previously described (Orandle et al., 1997). Briefly, lymphocytes were stained with anti-feline CD4-biotin (antibody CAT30A-bio) and anti-feline CD8-FITC (antibody CAT357), then streptavidin-phycoerythrin is added. Labeled cells were analyzed using a FACSan flow cytometer (Becton Dickinson, San Jose, CA) to determine the percentage of CD4+ and CD8+ cells.

2.6 Magnetic cell Sorting

At the time of euthanasia, thymus, blood and popliteal lymph nodes were aseptically collected, weighed and processed. Peripheral blood mononuclear cells (PBMCs) were enriched by Percoll discontinuous gradient centrifugation as described previously (Orandle et al., 1997). Thymus and lymph node tissues were weighed, and approximately cut into 5-mm cubes. Cubes were then minced and the lymphocytes were dissociated from the stroma by gentle pressing with a plunger from a 6-ml syringe. Aliquots of thymocytes, lymphocytes and PBMCs were divided into portions for RNA and DNA isolation. The remaining PBMCs were fractionated into B and T cell subsets using immunomagnetic methods as described previously (Orandle et al., 1997). Cells were processed with the MiniMACS high gradient magnetic cell sorting system (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. Briefly, 1×10^8 cells were incubated with PE-labeled appropriate mouse anti-CD4+ or anti-CD8+ and APC-labeled anti-CD79a (DakoCytomation California Inc., Carpinteria, CA) to sort B cells.

After incubation with antibody, cells were washed once with PBS and then incubated with colloidal supermagnetic beads conjugated with anti-PE or -APC. Cells were sorted by positive or negative selection and the cell purity was determined by FACS analysis. The purity of sorted cells for CD4⁺ and CD8⁺ cells were greater than 90% with B cells being greater than 95%.

2.7 Real-time polymerase chain reaction (PCR)

Genomic DNA was extracted from thymus, lymph node, PBMCs and sorted cells for the quantitative measurement of provirus DNA by the use of the QIAamp DNA Mini Kit (Qiagen Inc., Valencia CA). Real-time quantitative PCR was conducted using primers and Taqman probes designed from Primer Express Software (PE Applied Biosystems, Foster City, CA) for JSY3 gag and feline glycerol-dehyde-3-phosphate dehydrogenase gene (G3PDH). The sequences of these primers and probes were described previously (Norway et al., 2001). Real-time quantitative PCR was performed using the manufacturer's universal conditions (PCR Universal Master Mix; PE Applied Biosystems), 900 nM each forward and reverse primers, and 125 nM TaqMan probe in a 25- μ l PCR mixture volume. Samples were run in duplicate against serial dilutions of a cDNA standard containing the JSY3 gag sequence and feline (G3PDH). Proviral load was normalized to levels of G3PDH and expressed as copies per 100 ng cDNA. The relative FIV provirus was expressed as the ratio of FIV gag to G3PDH.

The level of early and late FIV transcripts were quantified by measuring the relative quantity of rev and gag transcripts, respectively, by real-time quantitative RT-PCR. Total RNA was extracted from the thymus, lymph node, PBMCs and sorted lymphocyte subpopulations using the RNeasy Midi Kit as recommended by the manufacturer (Qiagen Inc.). To measure the level of gag transcripts RT-PCR was performed using 0.5 μ g total RNA extracted from 3 $\times$ 10⁶ cells with the same gag primers and probe used to quantify the provirus described above. PCR was conducted using 100-200 ng of cDNA, 900 nM each of *gag*, *rev*, and G3PDH primer, and 125 nM of the TaqMan[®] probes. The level of rev transcripts were quantified using rev primers and a probe designed (Forward: CGG AAT GAA CTT CAA GAG GTA AAA CTA; Reverse: TCA TAT TCT TGA AGG CTT TCT TCC TT; Probe : 6FAM-CAG GTA AAA AGA AAA AAA GAC AAA-MGBNFQ) to anneal at the splice junction of exon 1 and 2 of rev mRNA. The relative FIV *gag* and *rev* gene transcription products were expressed as the ratio of FIV *gag* and *rev* to G3PDH.

2.8 Statistical analysis

A statistical software program (SigmaStat 3.0[®], SPSS Inc., Chicago, IL) was utilized for all data analyses. Hematologic profiles were analyzed by a one-way analysis of variance (ANOVA) for differences at each time point, and between infected animals as compared to uninfected age match controls. A one-way repeated measure analysis of variance (RM-ANOVA) was performed to compare an individual infected set of animals across the length of infection. Results are presented as means $\pm$ one standard deviation. Values with $P \leq 0.05$ were considered significant. Comparisons for proviral load and the relative levels of transcripts between infected animals, at the acute and chronic stage of infection, were performed by independent sample student's t-test (normal distribution and equal variance). Transformed variables that were not normally distributed or displayed unequal variances were compared using a non-parametric Mann-Whitney rank sum test. Results are presented as means $\pm$ one standard deviation. Values with a $P \leq 0.05$ were considered significant.

3. Results

3.1 Peripheral blood lymphocyte (PBL) profiles in acute and chronically infected kittens with wild type or Orf-A defective FIV

The hematological responses of cats, which include lymphocyte profiles of CD4⁺ and CD8⁺ cells, infected with JSY3 are documented (Norway et al., 2001; Yang et al., 1996). However, lymphocyte profiles that include B cells as well as comparative studies in cats infected with wild type or ORF-A deleted JSY3 at the acute and chronic stages of infections have not been carefully examined. Blood cells were analyzed to evaluate the effect on the different lymphocyte populations of PBLs during acute and chronic infections of an Orf-A-defective FIV as compared to the wild type. Lymphocytes were fractionated and examined at different time points to monitor for loss or expansion of cells associated with the progression of the FIV infection. In cats infected with wild type FIV (JSY3), there was a progressive inversion of the CD4:CD8 ratio until week 10 with the values being statistically different from uninfected control kittens ($P<0.05$) (Figure 1 A). The CD4:CD8 ratios for JSY3 infected animals at weeks 2 and 4 are lower than that of JSY3 Δ ORF-A ($P<0.05$). A statistically significant difference in the CD4:CD8 ratio was demonstrated for JSY3 Δ ORF-A at weeks 2 and 4 as compared to weeks 6, 8, 10, 12, 14 and 16 indicating a similar, yet less pronounced, inversion as was observed in the wild type infection. After week 8 the CD4:CD8 ratio leveled off with a slight increase at the chronic stage of infection for both mutant and wild type virus infection. From 2-8 weeks, the CD4:CD8 ratio for the animals infected with JSY3 was significantly lower than JSY3 Δ ORFA ($P<0.05$) (Figure 1 A), and by week 16 post-inoculation the CD4:CD8 ratios were similar for the 2 groups of infected cats. The inversion of the CD4:CD8 ratio was associated with a loss of CD4⁺ T cells in both groups of cats (Figure 1 B). This decline in absolute CD4⁺ T cells in cats infected with JSY3 was significant with P values <0.05 (weeks 2, 4, 8 and 12). A statistically significant difference was observed in absolute CD4⁺ cells of JSY3 Δ ORF-A at week 4 as compared to weeks 8, 12 and 16 suggesting that a reduction in CD4⁺ cells was also evident in animals infected with JSY3 Δ ORFA. The CD4⁺ T cell loss showed a trend that was greater for JSY3 infected cats for the first 10 weeks after infection compared to cats infected with JSY3 Δ ORFA and remained similar there after for the two groups indicating a delay in the onset of reduced peripheral blood CD4:CD8 ratio in JSY3 Δ ORFA infected animals. The absolute number of CD8⁺ T cells fluctuated during the 16-week course of FIV infection (Figure 1 C). There was no change in CD8⁺ cells in either group of infected cats compared to that of uninfected animals. Therefore, in an acute phase of FIV infection both groups showed a decrease in the CD4:CD8 cell ratio due to loss of CD4⁺ cells with the decrease being more pronounced in wild type JSY3 as compared to that of animals infected with JSY3 Δ ORF-A. In contrast, the chronic phase infections of both JSY3 and JSY3 Δ ORFA showed similar CD4:CD8 cell ratios. The CD4:CD8 cell ratios in the chronic stage of infection were decreased for both groups compared to the early acute stage of infection. Similarly we observed no increase in the absolute number of B cells in JSY3 infected animals as compared to uninfected ones (Figure 1D). Interestingly, there was statistically significant differences ($P<0.05$) in absolute B cell numbers in animals infected with JSY3 Δ ORFA at weeks 12, 14, and 16 as compared to weeks 4 and 6, while there was no significant changes for uninfected or wild type virus infected animals at those time points. Furthermore, at weeks 14 and 16 the results showed a trend that the absolute numbers of B cells were lower in animals infected with JSY3 Δ ORFA compared to uninfected controls. Taken together these results indicate a decline in the absolute number of B cells at the chronic stage in animals infected with JSY3 Δ ORFA.

3.2 Proviral load in lymphoid tissues and lymphocyte subpopulations in cats infected with wild type or Orf-A defective FIV

To determine whether there are differences in the proviral loads of animals infected with JSY3 or orf-A mutated FIV in acute versus chronically infected animals, viral DNA was quantified by real time PCR. DNA for real time PCR was extracted from thymus, lymph nodes, PBMCs, and blood lymphocyte subpopulations (CD4⁺ T cells, CD8⁺ T cells, and B cells) of cats infected with FIV at weeks 8 and 16. The quantity of provirus in these cell populations was determined using specific primers for the *gag* gene of JSY3. The proviral load at the acute phase (week 8) of infection in the tissues of thymus, lymph node and PBMCs was significantly less in cats infected with the Orf-A defective FIV as compared to that of the wild type infection ($P<0.05$) (Figure 2A). In contrast, the relative viral DNA in all three tissues showed minimal differences at the chronic stage (week 16) between JSY3 and JSY3 Δ ORFA infected animals (Figure 2B). Comparisons of proviral loads of JSY3 infected animals at weeks 8 and 16 revealed a reduction at week 16, whereas animals infected with the Orf-A defective FIV were found to have similar amounts of viral DNA at both time points in all the tissues examined. This reduction in JSY3 provirus load may have to do with the death or clearance of infected cells at week 16 (Crawford et al., 2001). The level of viral DNA was further examined in relation to tissue weight to confirm the observation that provirus DNA is significantly less in JSY3 Δ ORFA as compared to wild type in the acute phase of infection while remaining similar in the chronic phase of infection. As shown in Figure 3A proviral DNA per weight ratio in thymus is lower in JSY3 Δ ORFA infected animals as compared to that of JSY3 at week 8 ($P<0.05$), whereas at week 16 similar ratios were obtained for both infections, thus confirming similar amounts of proviral DNA in the wild type or Orf-A defective infected animals. Provirus DNA per weight ratio from the lymph node inclined to be comparable to that observed for thymus (Figure 3B).

The different lymphocyte subpopulations also showed a reduced trend of provirus DNA in CD4⁺, CD8⁺ and B cell populations of animals infected with JSY3 Δ ORFA at week 8 (Figure 4), implicating that in the acute phase of infection proviral load was reduced in cats infected with orf-A defective FIV compared to the wild type virus. Similar to the findings in the different tissues, the provirus load at week 16 was relatively lower than week 8 in CD4⁺ and CD8⁺ cells for animals infected with wild type JSY3. However, we found similar levels with high provirus loads at weeks 8 and 16 in the B cells of animals infected with JSY3. A previous study has also revealed a high level of infection with JSY3 in B cells at a chronic stage (Yang et al., 1996). In contrast, infection with JSY3 Δ ORFA showed a significantly lower amount of viral DNA in B cells at the chronic stage ($P<0.05$). Comparison of provirus copies at week 16 in CD4⁺ and CD8⁺ cells revealed minor differences between wild type and JSY3 Δ ORFA infected cats. Consequently, in the chronic stage of infection the differences in the relative amount of viral DNA is less pronounced between the JSY3 and JSY3 Δ ORFA infected groups as compared to the acute phase of infection with B cells being the exception. The decline in the differences between the two groups in the chronic phase of the infection appeared to be due to a decrease in the proviral load in cats infected with JSY3. In B cells, no reduction in viral DNA was observed in chronic stages of animals infected with JSY3.

3.3 Viral gene expression in animals infected with wild type or Orf-A defective FIV

In order to examine gene expression profiles in the absence of Orf-A in an acute or chronic FIV infection we examined early and late gene transcripts by monitoring for *rev* and *gag* mRNAs using the real time RT-PCR method. FIV *gag* and *rev* transcripts were quantified using RNA extracted from thymus, lymph node, and PBMCs collected from infected cats at weeks 8 and 16. We found that the relative amount of *gag* transcripts in the thymus, lymph nodes, and PBMCs were lower in cats infected with the Orf-A defective FIV as compared to cats infected with JSY3 virus at week 8 ($P<0.05$) (Figure 5A). Similarly the level of *rev* transcripts were significantly lower ($P<0.05$) at week 8 in the lymph node of cats infected with

JSY3ΔORFA (Figure 5C). At week 16 a reduction in gag transcripts of animals infected with JSY3ΔORFA was also observed in the thymus and lymph node compared to that of animals infected with JSY3 ($P<0.05$) (Figure 5B). A reduced trend in rev transcripts in all the tissues at week 16 and in the thymus and PBMCs at week 8 as well as gag transcripts in PBMCs at week 16 was also evident in JSY3ΔORFA infected animals (Figure 5B, C, D). In addition, we compared the level of rev and gag transcripts in the different subpopulations of lymphocytes isolated from PBMCs. As shown in Figure 6A and C animals infected with JSY3ΔORF showed significantly less gag and rev transcripts at week 8 in CD8⁺ cells ($P<0.05$). Viral transcripts in CD4⁺ and B cells at week 8 also tend to be reduced in animals infected with orf-A defective virus compared to animals infected with wild type virus. At week 16 B cells showed a significant ($P<0.05$) decrease in the level of viral transcripts in animals infected with JSY3ΔORFA (Figure 6B and D). In CD4⁺ cells of animals infected with the orf-A defective virus the level of rev transcripts at week 16 was also significantly lower ($P<0.05$) than that of wildtype. The level of gag transcripts in CD4⁺ cells at week 16 showed a trend of lower values in animals infected with the orf-A defective virus. In CD8⁺ cells, however, we observed no significant differences in gag transcripts at week 16 between animals infected with JSY3 and JSY3ΔORF, whereas the relative level of rev transcripts in CD8⁺ cells at week 16 in animals infected with JSY3ΔORFA tends to be reduced. Comparisons of viral transcripts in B cells between weeks 8 and 16 showed similar levels of gag transcripts in both the mutant and wild type virus infection (Figure 6 A and B). For both JSY3 and JSY3ΔORF-A infected animals the difference in gene expression in the B cells was more pronounced than in the T cells at week 16, similar to what was observed for the proviral load. These results demonstrated a general trend that the levels of transcripts were reduced in both acute and chronic infection in animals infected with JSY3ΔORF, which subsequently may contribute to delay in virus replication and the onset of pathogenesis in the infected animals.

4. Discussions

In this report we compared the FIV proviral load and gene expression in tissues and lymphocyte subpopulations in acute and chronically infected cats. We observed that the proviral load in the animals infected with ORF-A defective virus during the acute stage of infection was significantly less than that of the wild type. Interestingly, the level of proviral load in animals infected with orf-A mutated FIV during chronic infection is similar to that of the wild type due to a decline in the amount of wild type viral DNA in the chronic stage of infection. The only exception was in B cells where provirus burden was equivalent in the acute and chronic stages in animals infected with the wild type virus, while viral DNA was significantly less in animals infected with JSY3ΔORF-A. Viral gene expression for both time points were less in animals infected with Orf-A defective FIV than that of the wild type infection. In the chronically infected animals, the observed difference in B cell viral gene expression between wild type and orf-A defective virus was more than that which was found for T cells.

Infection studies by Yang et al at different time points with JSY3 revealed that during the acute phase of infection, FIV is predominantly detected in the CD4⁺ subset of PBMCs and the proviral load then shifts to CD8⁺ and B lymphocytes at a later stage of infection (Yang et al., 1996). In the acute phase of infection, JSY3 provirus was found only in the CD4⁺ lymphocyte subset. Contrary to this report our studies show equivalent amounts of provirus in all of the lymphocyte subpopulations during the acute stage of infection. This discrepancy between our results and the previous report may be due to differences in the times that samples were obtained during the acute stage of infection. In the Yang et al studies, samples were taken at two weeks post infection to determine provirus burden. It is possible that at week eight the shifts toward CD8⁺ and B cell infection may have begun. Additionally, there is a difference in the age of the animals used in the two studies. This study used one-day-old kittens whereas Yang et al used adult cats. The provirus load in B cells at both stages of infection was equivalent while

there was a decreased DNA level in CD4+ and CD8+ cells in the chronic stage infection. In chronic stages the greatest provirus burden was detected in B cells confirming the previous report (Yang et al., 1996). In animals infected with our Orf-A defective FIV we observed the provirus burden remained similar in all lymphocyte subpopulations implicating that no shift in provirus load had taken place to B cells in the chronic stage of infection.

The mechanism by which Orf-A enhances virus replication and its role in pathogenesis is not known. Although at low levels, ORF-A is capable of transactivating gene expression directed by the FIV promoter (De Parseval and Elder, 1999; Sparger et al., 1992). In established cell lines the FIV LTR has a strong basal promoter activity. The reason why a transactivator gene exists in FIV while the LTR contains a strong promoter is not completely understood.

Interestingly, however, no information is available about basal promoter activity of the FIV LTR in primary cells (PBMCs). The molecular clone FIV-p34 is able to productively infect CrFK cells with a high titer virus production, but not PBMCs (Phillips et al., 1990; Sparger et al., 1994). FIV-p34 is a natural mutant that has a stop codon in the middle of orf-A (Talbot et al., 1989). When the stop codon is converted to a tryptophan codon, FIV-p34 is capable of replicating in feline PBMCs, implicating ORF-A as one of the important factors for replicating in primary cells (Waters et al., 1996). The mechanism by which the converted ORF-A allows virus replication in PBMCs is not known. The minor transactivation by ORF-A, however, may not account for the level of virus restriction with the Orf-A defective FIV. Similar to the accessory genes of HIV, Orf-A may have several functions, which promotes virus replication and the establishment of an infection. The finding that Orf-A is required for virus particle formation/virus infectivity and, similar to Vpr, induces cell cycle arrest at the G2 phase of the cell cycle supports this notion (Gemeniano et al., 2003; Gemeniano et al., 2004). Our *in vivo* studies in the acute phase showed a significant reduction in the level of viral transcripts in all the different lymphocytes of animals infected with the Orf-A defective virus. The low level viral transcripts in animals infected with Orf-A defective virus may reflect the reduction in provirus load and can potentially be related to the problem of virus particle formation and/or efficiency of virus infectivity. Our studies also reveal that gene expression is active in all tissues and lymphocyte subpopulations of the animals infected with wild type virus at the chronic phase, with the highest amounts of viral transcripts being in B cells. Yang et al suggested that the progressive decline in the number of CD4+ cells while CD8+ and B cells remain normal imply that FIV infection is latent and noncytolytic in CD8 and B cells (Yang et al., 1996). However, our findings establish that viral replication at the level of gene expression is not latent in CD8+ and B cells in infected animals at week 16. Whether the active transcription in CD8+ and B cells in the chronic phase leads to cytolytic infection remains to be determined.

The reason for high proviral load and viral transcripts in B cells during the chronic stage compared to the other lymphocyte subpopulations in animals infected with wild type virus is not clear. Interestingly, the number of absolute B cells in animals infected with Orf-A defective virus at weeks 4 and 6 were significantly higher than at weeks 12, 14 and 16, whereas the number of B cells remain without significant changes from week 4 to 16 in animals infected with wild type virus (Figure 1). The mean value for absolute B cell numbers at week 16 for animals infected with JSY3 and JSY3ΔORF-A are 2,954 cells/ul and 768 cells/ul, respectively. These values in cell number may reflect our observation of higher provirus load and gene expression at week 16 with JSY3 compared to JSY3ΔORF-A. However, examining the CD8+ cell mean values at week 8 for JSY3 (406 cells/ul) and JSY3ΔORF-A (326 cells/ul), which had an equivalent number of cells, revealed a significantly reduced gene expression with the orf-A defective virus. The provirus load in CD8+ cells at week 8 also showed a trend that is lower for JSY3ΔORF-A. Similarly, CD4+ cells where there was no significant difference in cell numbers at week 8 (with mean values 276 cells/ul for JSY3 and 624 cells/ul for JSY3ΔORF-A) showed provirus load and gene expression in animals infected with orf-A defective virus tend to be lower where the mean value cell number is higher. Therefore, the

decline in the absolute B cell number may not explain the differences in provirus loads and level of virus transcripts observed. The reason for the decrease in the B cell number is not clear and warrants further investigations. Whether Orf-A has an important contribution to the gradual shift from the high burden in CD4⁺ cells during acute phase infection to the asymptomatic stage, which the infection predominates in B cells remains to be determined.

Previous comparative infection studies between wild type and orf-A defective FIV revealed a restriction in virus replication in PBMCs when the orf-A gene is mutated (Dean et al., 1999; Pistello et al., 2005; Pistello et al., 2002; Sparger et al., 1994). Furthermore, an Orf-A defective FIV has been implicated as a potential live attenuated vaccine (Pistello et al., 2005). Interestingly, Orf-A defective mutant virus replication is not altered in monocyte derived macrophages (MDM) compared to wild type virus (Pistello et al., 2005; Pistello et al., 2002), indicating that Orf-A is not necessary in MDM or the requirement for Orf-A may be compensated with an MDM specific factor. Although at reduced levels our results show that the orf-A defective virus replicates in lymphoid tissues as well as in the different lymphocyte subpopulations isolated from PBMCs. This discrepancy between our and previous reports may have to do with the age of the animals; previous studies used adult animals. Nef-deleted SIV in monkeys replicate and cause disease in neonates where as in adults the mutant virus is highly attenuated (Baba et al., 1995; Kestler et al., 1991). Similarly the function of Orf-A in virus replication and pathogenesis may be influenced by the age of the animal. In support of this notion our previous studies in neonate cats demonstrated that the formation and extent of thymic lymphoid follicular hyperplasia were similar in JSY3 and JSY3 Δ ORF-A infected cats as measured by anticytokeratin immunohistochemistry and flow cytometry for percent pan T-negative and immunoglobulin G-positive cells within the thymus (Norway et al., 2001). These observations suggest that the use of an orf-A defective FIV for vaccine purposes needs to be scrutinized by intensive experimental evidence for safety before considering its application.

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Figure 1.

Comparison of hematologic profiles in animals infected with JSY3 and JSY3ΔORF-A. Peripheral blood mononuclear cells were isolated biweekly from infected animals and cell counts were determined by percentage of cells by FACS, complete blood counts and differentials. The number of uninfected animals per time point ranged from 4 to 10 except for week 14 where only 2 uninfected controls were used. Mean CD4+/CD8+ ratios were established from the percentage of CD4+ and CD8+ cells obtained from flow cytometry. Each data point represents the mean of each ratio of 6 cats for JSY3 and 10 cats for JSY3ΔORF-A, with the error bars showing standard errors of the means. Data from age matched uninfected controls and infected cats were compared by use of a one-way ANOVA. A one-way repeated measure analysis of variance (RM-ANOVA) was performed to compare an individual infected set of animals across the length of infection. Values with a $P \leq 0.05$ were considered significant.

Figure 2.

Provirus load in the thymus (Thy), lymph node (LN) and peripheral blood mononuclear cells (PBMCs) in FIV infected kittens at week 8 (A) and 16 (B). The kittens were infected at birth with 10^4 TCID₅₀ of JSY3 (n=6) or JSY3ΔORF-A (n=10). The thymus, lymph nodes and blood were harvested from kittens at 8 weeks (JSY3, n=3 and JSY3ΔORF-A, n=4 kittens) and 16 weeks (JSY3, n=3 and JSY3ΔORF-A, n=6) post-infection. The relative proviral loads were determined by real-time PCR using primers specific for the gag gene. Proviral copy numbers were standardized to G3PDH and expressed as copies per 100 ng of genomic DNA. The relative FIV provirus was expressed as the ratio of FIV gag to G3PDH. *The gag proviral load in kittens infected with JSY3ΔORF-A was significantly ($P < 0.05$) less than in kittens infected with JSY3 (WT).

Figure 3.

FIV provirus load per tissue weight. The relative gag gene copy number obtained by real time PCR from thymus (A) and lymph node (B) was calculated per gram tissue to determine provirus load per weight ratio. *The relative gag proviral load per gram tissue in kittens infected with JSY3ΔORF-A was significantly ($P < 0.05$) less than in kittens infected with JSY3 (WT). **The values between JSY3 (WT) week 8 and JSY3 (WT) week 16 are significantly different ($P < 0.05$).

Figure 4.

FIV provirus load in lymphocyte subpopulations at 8 week (A) or 16 weeks (B) post-infection. Blood was collected at 8 weeks or 16 weeks from kittens infected with JSY3 or JSY3ΔORF-A. CD4⁺, CD8⁺ or B cells were purified from the peripheral blood mononuclear cells by immunomagnetic selection method. Provirus load in each lymphocyte population was determined by real-time PCR assay using primers specific for the gag gene. RNA samples (0.5 ug) were reverse transcribed and PCR conducted using 100-200 ng cDNA. The relative FIV *gag* and *rev* gene transcription products were expressed as the ratio of FIV *gag* or *rev* to G3PDH. *The *gag* proviral load in kittens infected with JSY3ΔORF-A was significantly ($P < 0.05$) less than in kittens infected with JSY3 (WT).

Figure 5.

Real time PCR analysis of viral transcripts in thymus (Thy), Lymph node (LN) and peripheral blood mononuclear cells (PBMCs) of kittens infected with JSY3 or JSY3ΔORF-A. RNA for PCR from each tissue was obtained at week 8 (A and C) or 16 (B and D) post-infection. Real time PCR was performed using primers specific for gag (A and B) or rev (C and D) transcripts. **gag* or *rev* expression in kittens infected with JSY3ΔORF-A was significantly ($P < 0.05$) less than in kittens infected with JSY3 (WT).

Figure 6.

Analysis of FIV transcripts in lymphocyte subpopulations by real time PCR. PBMCs were isolated from JSY3 or JSY3ΔORF-A infected kittens at week 8 (A and C) or 16 (B and D) for purification of lymphocyte subpopulations. CD4+, CD8+ or B cells were purified by immunomagnetic selection method and RNA isolated for real time PCR analysis. Primers specific for gag (A and B) or rev (C and D) transcripts were used for real time PCR analysis. *gag or rev expressions in kittens infected with JSY3ΔORF-A was significantly ($P < 0.05$) less than in kittens infected with JSY3 (WT).