Detection of Proviruses and Viral RNA in the Early Stages of Feline Immunodeficiency Virus Infection in Cats: A Possible Model of the Early Stage of HIV Infection

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Abstract: Feline immunodeficiency virus (FIV) infection in cats has been reported to be a useful animal model for human AIDS studies, especially in the early stages of infection. We examined the temporal changes in provirus detection in peripheral blood mononuclear cells (PBMC) and the distribution of FIV-DNA and RNA in feline tissues by the polymerase chain reaction at 10, 35, 70 days after intravenous inoculation of FIV. Viral DNA in the PBMC was detected three to four weeks after infection and its fluctuation was demonstrated for the first time. Ten days after infection, before seroconversion, proviruses were detected only in the mesenteric lymph nodes and intestines. At 35 and 70 days after infection, after seroconversion, proviruses were detected in most lymphoid organs and the salivary glands, but the expression of FIV-RNA was limited to the thymus at 70 days after infection. These results show that FIV-RNA is transcribed from proviral DNA exclusively in the thymus at this stage. We suggest that the quantitative changes in detectable proviruses in the PBMC depend on the relation between the decrease in infected cells caused by cytolytic T lymphocytes and/or apoptosis and their increase caused by the release of a new supply of lymphocytes from the thymus.

Key words: FIV, HIV, polymerase chain reaction, provirus

Introduction

Feline immunodeficiency virus (FIV) is a T cell-tropic lentivirus isolated from cats [33]. Coinfection with this virus and feline leukemia virus causes symptoms resembling those of human AIDS. FIV resembles the human immunodeficiency virus (HIV) biologically and biochemically [17, 22, 26, 39]. Spontaneous or experimental infection [40, 44] of cats with FIV results in selective depletion of CD4+ T cells [25], leading to opportunistic infections [1, 2, 11]. The FIV can infect macrophages [5] and it is neurotropic [10, 19]. Because of these features, cats infected with FIV have been attracting close attention as an animal model of

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HIV infection. Clarification of the kinetics of the HIV in the early infection stage seems to lead to treatment. To this end, studies of animals early after FIV infection may provide us with useful information.

Recent studies have shown that HIV continues to replicate in lymphoid tissues during the whole clinical course of HIV infection, and that CD4+ T cells, macrophages and follicular dendritic cells (FDC) serve as HIV reservoirs [12, 27, 28]. Similar findings have also been reported for FIV [41]. Furthermore, the clearance of HIV from peripheral blood and the turnover of CD4+ T cells in HIV infections have recently been understood from the analyses of responses to several anti-viral agents [15, 43]. A decrease in CD4+ cells has been attributed to apoptosis which occurs via two mechanisms [13, 30], (1) an increase in physiological apoptosis due to the activation of effector cells, including cytotoxic T lymphocytes (CTL) following viral infection, and (2) an increase in apoptosis due to abnormal signaling caused by viral infection. These reports also suggest that lymphoid tissue forms an effective immune system against HIV infection by combining trapping of virions by the FDC network with sequestering infected CD4+ T cells in the lymph nodes. We attempted to clarify the dynamics of FIV in the early stages of infection by analyzing quantitative changes of peripheral blood proviruses in the very early stages of FIV infection in cats, and by checking the tissue distribution (especially in the primary lymphoid organs and the secondary lymphoid organs) of proviruses and FIV-RNA by means of the polymerase chain reaction (PCR).

Materials and Methods

Animals: Specific pathogen-free (SPF) cats were purchased from IFFA CRED, France and were caged individually in the departmental animal facilities isolated from each other.

Infection of cats with FIV: To study antibody response and kinetics of proviral DNA in PBMC, six SPF cats, 24 to 30 weeks of age and of random sex selection, were inoculated intravenously with 3 × 10^3 TCID_{50}/ml/body (group C-1 ~ C-6). The TCID_{50} was calculated by MYA-1 (a feline CD4+ lymphoblastoid cell line [24]) infected with FIV (KYO-1 strain) isolated from a domestic cat in Tokyo, Japan, and the cell-free viruses were obtained from MYA-1 infected with the KYO-1 strain. To study the virus distribution in cat tissues, additional 6 SPF cats, 24 to 30 weeks of age and of random sex, were inoculated intravenously with 3 × 10^3 TCID_{50}/ml/body (group A-1 ~ A-3) or with 3 × 10^2 TCID_{50}/ml/body (group B-1 ~ B-3). At weekly intervals after infection, samples of the venous blood of the cats in group C were collected. Tissue samples (lympho-adenoid, brain, liver, lung, intestine and femoropatellar joint) were collected at necropsy and frozen immediately in liquid nitrogen 10 days (A-1 and B-1), 35 days (A-2 and B-2) and 70 days (A-3 and B-3) after infection.

Radiolabeling of cells: Cells of MYA-1 productively infected with FIV (KYO-1 strain) in 75 cm^2 Falcon flasks were incubated for 15 min at 37°C in methionine-free RPMI 1640 medium (GIBCO). The cell cultures were then incubated for 18 hr in 20 ml of the same medium containing 2.5 mCi/ml (35)S methionine (sp. act. 1200 Ci/mmol, NEN), and the cells were centrifuged and washed with phosphate buffered saline (pH 6.4), and lysed with 5 ml of lysis buffer containing 0.5 M Tris-HCl (pH 8.0), 0.05 M NaCl, 0.5% NP-40 and 1 mM pAPMSF (Sigma). Before use the cell lysates were clarified by centrifuging at 100,000 g for 30 min at 4°C and the pellets were discarded.

Immunoprecipitation of labeled cell extracts: Fifty µl of labeled cell lysate, 100 µl of lysis buffer and 5 µl of test plasma were mixed in a microcentrifuge tube and incubated overnight at 4°C. Then 100 µl of a suspension of 50% Protein A-Sepharose CL-4B beads (Pharmacia) in lysis buffer was added to each tube and mixed for 15 min at room temperature. The antigen-antibody complex bound to Protein A-Sepharose beads was collected by centrifugation followed by washing three times with lysis buffer, and then washed twice with 10 mM Tris-HCl (pH 8.0). The final pellet was resuspended in 40 µl of SDS-PAGE loading buffer and heated for 5 min at 100°C. After centrifugation, the supernatant was applied to SDS-PAGE gel for electrophoresis, followed by fluorography with 1 M sodium salicylate as an enhancer of the fluorography and an exposure at −80°C to Kodak XAR-5 film.

DNA and RNA extraction: Peripheral blood mononuclear cells (PBMC) and plasma samples were separated by the Ficoll - Conray procedure. All samples
Tissue distribution of FIV DNA and RNA

were stocked at −80°C until assayed. For DNA extraction, PBMC (2 × 10⁶ cells) and tissues minced on ice were dissolved in 200 μl of DNA lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate, and 200 μg of proteinase K per ml), incubated for 2 hr at 55°C, and extracted with phenol followed by phenol-chloroform (1:1), and finally with chloroform-isooamyl alcohol (24:1). DNA was precipitated with ethanol and then dissolved in TE buffer (10 mM Tris-HCl [pH 7.5] and 1 mM EDTA).

The RNA was extracted by the single-step method with acid guanidinium thiocyanate-phenol-chloroform described by Chomczynski and Sacchi [7]. To remove the contaminant DNA, 2 μg of extracted RNA was incubated with 10 U of RNase-free DNase I (Promega), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 6 mM MgCl₂ (total reaction volume of 30 μl) at 37°C for 15 min and it was then heated at 95°C for 5 min to stop the reaction.

As the negative control of viral DNA and RNA uninfected MYA-1 was used, and as the positive control, MYA-1 infected with KYO-1 was used.

Reverse transcription (RT) and polymerase chain reaction (PCR): The primers and probe used were derived from the sequence of the FIV gag region: sense primer, GGCCATTAAGAGATGATGA [nucleotides No. 666 to 685]; antisense primer, ATGGCCATATCAAATTTCCCT [nucleotide No. 882 to 863]; and probe, GTAACATACGGACGAGACCCAGGTGATATACCCAGAGCT [nucleotide No. 763 to 802]. DNA amplification was performed at 30 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min) and the final reaction was at 72°C for 5 min in a total volume of 50 μl containing 0.5 μg of the sample, 100 ng each of the primers, 400 μM deoxynucleoside triphosphates (dNTPs), 0.25 U of Taq polymerase (Takara, Japan), 1.5 mM MgCl₂, and Taq polymerase buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.01% gelatin). Reverse transcription was performed with 10 μl DNase I-treated RNA samples or RNA controls in a total volume of 25 μl containing 6 mM MgCl₂, 400 μM dNTPs, 10 mM dithiothreitol (DTT), 50 ng of the antisense primer, 5 U of RNasin (Promega), 2 U of Moloney murine leukemia virus RTase (Pharmacia), and Taq polymerase buffer. After incubation at 37°C for 1 hr, 100 ng each of the primers, 400 μM dNTPs, 0.5 U of Taq polymerase and Taq polymerase buffer were added to the mixtures (total reaction volume of 100 μl). Reverse transcribed viral DNA was amplified by 40 cycles of PCR.

Amplified DNA fragments were separated by electrophoresis in 3% NuSieve GTG agarose-1% agarose gel transferred to nylon membrane (Paul) and hybridized overnight at 56°C with the biotinylated probe prepared by 3'-labeling kits with terminal transferase (Enzo, USA). The hybridized DNA was detected by using the chemical luminescence from the reaction of dioxetane as a substrate and alkaline phosphatase (Sumitomo Kinzoku, Japan).

The density of the band of PCR products of FIV-DNA was analyzed with a densitometer semi-quantitatively and defined at level 1, 2 or 3.

Results

Figure 1 shows the dynamics of the provirus in the PBMC from six cats (C-1 to C-6) during the first 15 weeks after infection. At weeks 3, 10 and 15, proviruses were detected in all cats. In cat C-6, proviruses were first detected at week 2, and in the other five cats they were first detected at week 3. In cats C-4 and C-5, the proviruses were not detected at weeks 12 and 4, respectively; in C-2, they were not detected at weeks 12 and 13; in C-3, they were not detected at weeks 5, 8 and 11; and in C-1, they were not detected at weeks 4 to 9 and at week 12. The provirus was continuously detected throughout the entire study period in only one cat (C-6). Figure 1 also shows semi-quantitatively the density of the band of PCR products of FIV-DNA. The density varied and did not show any particular tendency to increase, although it differed among individual animals. These results were identical to those obtained in a previous pilot study. When we carried out this experiment in duplicate, with the same samples, the results were reproducible.

Figure 2 shows the duration of plasma anti-FIV antibody levels in the 6 cats (C-1 to C-6). The antibody levels, determined by the radioactivity immunoprecipitated by preinfected fertile plasma subtracted from the total radioactivities of immunoprecipitates, increased significantly from week 4 (P<0.01). The antibody levels continued to increase until week 10 and reached a plateau. Electrophoresis by SDS-PAGE showed that
all cats had expressed antibodies to p24, gp42 and gp120 (data not shown). A similar antibody profile after week 4 was also seen in C-2, whose rise in antibody levels was lower than that in the other cats. These findings from PCR and antibody analyses (i.e. the detection of the proviruses at week 3, one week earlier than the appearance of serum antibodies, and the detection of antibodies) clearly showed that FIV infection of peripheral blood T-cells occurred in the very early stage after the entry of FIV into the cat’s body.

Tables 1-a, b show the test results in the PBMC and tissues. On the 10th day after infection, the proviruses in the PBMC were not detected in cats A-1 and B-1. At 35 and 70 days, the proviruses in the PBMC were detected in two cats, respectively. On the 10th day after infection, A-1 infected with KYO-1 (3 x 10^3 TCID_{50}/ml) had proviruses only in the mesenteric lymph nodes and the intestine, and proviruses were not detected in other tissues of A-1 or in any tissues of B-1 infected with 3 x 10^2 TCID_{50}/ml with KYO-1. At 35 and 70 days, however, proviruses were detected in many tissues, including the primary lymphoid organ, secondary lymphoid organ and intestines. In addition, their concentration was particularly high in the thymus, bone marrow, spleen and mesenteric lymph nodes when semi-quantitative analyses of these tissues were performed by PCR with 5-fold serial dilutions of template DNA and dot blot hybridization (data not shown). No proviruses were detected in the brain. These results showed that FIV infection of lymphoid tissue had occurred within 35 days, although the infectious virus level was still low (FIV-RNA was not detected in these plasma, data not shown), and that it had progressed to a persistent infection. The results also suggest that, although FIV is a neurotropic virus, it did not pass through the blood-brain barrier, at least during the first 70 days of infection.

With the same sample, we then examined the distribution of FIV-RNA in tissues by RT-PCR. Figure 3 shows that at 70 days FIV-RNA was detected only in the thymus (A-3: Lane 4, B-3: Lane 4), but not in other tissues. On the 10th day, and at 35 days, FIV-RNA was not detected in any tissues or PBMC (data not shown).
Fig. 2. The time course of anti-FIV antibody levels in feline plasma. The antibody levels are indicated by the total radioactivity of the immunoprecipitation by using infected cat serum minus that of preinfected serum. Week 0 is the time of inoculation with FIV. The curve represents the correlation curve, and the coefficient of correlation is $R^2=0.953$. Data show the mean ± standard deviation among 6 cats (C-1 ~ C-6). From 4 weeks after infection, the antibody levels increased significantly ($P<0.01$).

□: C-1, ○: C-2, △: C-3, ■: C-4, ●: C-5, ▲: C-6.

Table 1-a. Distribution of FIV-DNA in feline tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10 days</th>
<th>35 days</th>
<th>70 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-1</td>
<td>B-1</td>
<td>A-2</td>
</tr>
<tr>
<td>PBMC</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tonsils</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Thymus</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Parotid L. N.</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Submandibular L. N.</td>
<td>-</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Pulmonary L. N.</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Mesenteric L. N.</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cervical L. N.</td>
<td>-</td>
<td>-</td>
<td>NT</td>
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<tr>
<td>Liver</td>
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</tr>
</tbody>
</table>

- : negative, + : positive, NT: not tested, L. N.: lymph nodes.
PBMC: peripheral blood mononuclear cells.

Table 1-b. Distribution of FIV-DNA in feline tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10 days</th>
<th>35 days</th>
<th>70 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-1</td>
<td>B-1</td>
<td>A-2</td>
</tr>
<tr>
<td>Lungs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Intestine</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Femoropatellar joint</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bone marrow (femur)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bone marrow (sternum)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Temporal lobe of C.</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Occipital lobe of C.</td>
<td>-</td>
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</table>

Discussion

We detected proviruses in the PBMC at week 3 after the infection of cats with FIV, similar to the reports by Hohdatsu et al. [16], Lawson et al. [20], and Matteucci et al. [23]. But during weeks 3 to 15 their detection was irregular. The duration of the detection of proviruses during the early stages of infection has not been reported. We found that antibody expressions were noticeable at week 4 and increased until week 10 when they reached a plateau, as was reported by O'Connor et al. [26], Hosie et al. [17] and Rideout et al. [35]. Many reports have described the detection of FIV by PCR following experimental or spontaneous infection [8, 16, 20, 34]. Our results confirm the findings of Dandekar et al. [8] which showed that some cases were antibody positive, but PCR (gag gene) negative, and the findings of Hohdatsu et al. (1992) which showed that 5 out of 12 seropositive cats were negative in single PCR, but they did not report that the levels of detectable proviruses in the PBMC fluctuate in the early stages of FIV-infection.

The dynamics of the proviruses in the PBMC cannot be explained unless the following three factors are taken into account: (1) viral mutation, (2) sites of virus production, and (3) immunological purge or death of infected cells. As pointed out by Greene et al. [14], viral mutation seems to be unlikely to be involved, because the gag region used for our PCR seldom undergoes mutation.

We analyzed the site of virus production in detail by checking the tissues for proviruses and FIV-RNA con-
cerning the following points: (a) early stages of infection, (b) antibody producing stages, and (c) antibody positive and PCR positive stages as shown in group C. These analyses revealed that proviruses were positive in many lymphoid tissues, but FIV-RNA was positive only in the thymus at 70 days. These results suggest that FIV-DNA is transcribed within thymic cells at 70 days, as reported by Pantaleo et al. [27] in HIV infection, giving rise to persistent infection of the thymus at week 10. Beebe et al. [3] injected the whole blood of a Petaluma strain-infected cat into other cats intraperitoneally. Four weeks later, they detected FIV-DNA and RNA in the thymus cortex of the injected cats by in situ hybridization. Diehl et al. [9] showed that severe thymic atrophy was appeared at week 4 after infection with FIV resourced in the acute phase of infection. The difference between their study [3, 9] and ours in the timing of FIV-RNA detection in the thymus seems to be attributable to differences in viral strains, different routes of infection and the use of a cell-free virus, but both reports coincide in detecting viral RNA in the early stages of the infection. In fact, the infected cats in their study developed acute illness, whereas our cats remained healthy, despite antibody increase. Bonyhadi et al. [4] obtained similar results on HIV infection in the SCID-hu mouse. Some investigators have reported on HIV infection of the thymus following the onset of AIDS [18]. Infection of the thymus with HIV has mainly been shown from in vitro experiments with thymocyte cell lines [36, 37, 42]. Although the thymus has been attracting close attention as a possible virus reservoir [4, 21, 27], no reports on the role of the thymus in the early symptom-free stages of infection have been published. If the results of this study are combined with the results of Patterson et al. [32], we can say that persistent HIV infection occurs earlier in the thymus than in any other lymphoid tissue and that the thymus is a site of virus production, although the timing of infection of the thymus varies depending on the amounts of virus invading the body or on the route of virus transmission.

Pantaleo and Fauci [30] recently gave an overview of the purge or death of infected cells, and Finkel et al. [13] described a detailed study of apoptosis. Cao et al. [6], Ho et al. [15], Pantaleo et al. [31], and Wei et al. [43] mathematically studied the clearance of HIV and the turnover of infected cells in patients with chronic HIV infection. They found that both the viral clearance and the turnover of infected cells are rapid. Pantaleo et al. [29, 30] and Cao et al. [6] showed that high CTL activity is needed for the clearance of the HIV. Song et al. [38] showed the presence of FIV-specific CTL at weeks 7–9 of FIV infection. Therefore, in the early symptom-free stages of FIV infection, the cycles of appearance and disappearance of proviruses in PBMC are caused by the balance between the decrease and increase in infected cells by the following mechanisms: (1) the proviruses in PBMC decrease due to the activation of CTL following virus infection of the thymus cortex, to immunological purge of infected cells or to an increase in cell death due to an increase in normally programmed apoptosis; (2) the reproduction of infected CD4+ cells occurs in PBMC because the primary lymphoid tissue serves as a reservoir which continuously supplies virus-infected cells to the peripheral blood.

FIV also resembles HIV in its neuropathy. Since the transfer of FIV into the brain did not occur during the first 10 weeks of infection in our study, it is plausible to imagine that the brain is not infected with HIV in the early stages of HIV infection. Although early detection of HIV infection allows early treatment, care is needed when PCR is used to assess the therapeutic efficacy in cases of HIV infection, especially when single PCR is used to diagnose HIV infection.

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References


