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Plasma Viral RNA Load Predicts Disease Progression in Accelerated Feline Immunodeficiency Virus Infection

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Viral RNA load has been shown to indicate disease stage and predict the rapidity of disease progression in human immunodeficiency virus type 1 (HIV-1)-infected individuals. We had previously demonstrated that feline immunodeficiency virus (FIV) RNA levels in plasma correlate with disease stage in infected cats. Here we expand upon those observations by demonstrating that plasma virus load is 1 to 2 logs higher in cats with rapidly progressive FIV disease than in long-term survivors. Differences in plasma FIV RNA levels are evident by 1 to 2 weeks after infection and are consistent throughout infection. We also evaluated humoral immune responses in FIV-infected cats for correlation with survival times. Total anti-FIV antibody titers did not differ between cats with rapidly progressive FIV disease and long-term survivors. These findings indicate that virus replication plays an important role in FIV disease progression, as it does in HIV-1 disease progression. The parallels in virus loads and disease progressions between HIV-1 and FIV support the idea that the accelerated disease model is well suited for the study of therapeutic agents directed at reducing lentiviral replication.

Feline immunodeficiency virus (FIV) infection in cats results in a disease syndrome similar to that caused by human immunodeficiency virus type 1 (HIV-1) infection in humans. Both FIV and HIV-1 infections are characterized by progressive $CD4^+$ T-cell loss and lymphoid depletion (19, 26), immunologic abnormalities (1, 23, 40), and susceptibility to opportunistic infections (9, 18, 27). Disease stage progressions of FIV and HIV-1 are also similar, beginning with a transient acute-phase illness followed by a prolonged asymptomatic period and a terminal symptomatic phase (6, 7, 9, 17, 27, 34). These similarities have led to the study of FIV infection in cats as an animal model for HIV-1 infection.

Virus expression levels have been shown to correlate with disease stages in FIV and HIV-1 infections (7, 28–31). A very high viral load in plasma is associated with acute infection, and then viral RNA levels decline but remain significant during the asymptomatic phase and increase again in the terminal stage (2, 7, 12–14, 29, 31). More recently, HIV-1 RNA levels in peripheral blood mononuclear cells from asymptomatic individuals have been shown to be predictive of the rapidity of disease progression (22, 33).

We have developed a rapid and consistent FIV disease model (8) which accelerates the prolonged and variable disease course frequently associated with experimental FIV infections (26). Here we demonstrate that cats with rapidly progressive FIV typically have plasma RNA loads 1 to 2 logs greater than those of long-term survivors and that this difference is present early in infection and persists throughout the course of disease. These findings indicate that, as with HIV-1, FIV replication plays an important role in disease progression, and they enhance the utility of the FIV model in assessing AIDS therapy strategies aimed at reducing replicative virus burden.

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MATERIALS AND METHODS

Animals. Eight-, 12-, and 13-week-old cats from a specific-pathogen-free breeding colony at Colorado State University were inoculated by intravenous injection of cell-free plasma or whole blood from cats infected with FIV subgroup C PGammer (FIV-C-PGammer) (CABC padyOOC). Blood was collected from infected cats and uninfected control animals for plasma and serum harvesting at weekly intervals. Clinical symptoms, hematologic alterations, and necropsy findings for these animals have been described in a previous paper (8). Cats are referred to as rapid-progressor or long-term-survivor animals on the basis of the rapidity of disease progression and mortality following FIV infection. Long-term-survivor animals were asymptomatic at 38 weeks (phase 3 cats) or 29 weeks (phase 4 cats) postinfection (p.i.), when the study was terminated.

Virus source and infections. The passage history for FIV-C-PGammer has previously been described (8). Briefly, the FIV-C-PGammer virus source was plasma from a clinically symptomatic FIV-infected cat obtained during studies designed to collect and subtype FIV field isolates (35). Source animal plasma of unknown titer was used to inoculate a single 8-week-old specific-pathogen-free cat (phase 1 of the study). During the acute phase of infection, plasma was collected and pooled to serve as a virus source for subsequent infections. This plasma pool was used to inoculate four 8-week-old cats (phase 2 of the study) with 2 tissue culture infective doses each of FIV as determined by plasma culture. Terminal plasma (4 weeks p.i.) from a phase 2 animal, cat 3227, was used as the source virus for the infection of 12-week-old phase 3 cats. The 12 phase 3 cats were divided into four groups, each of which received log dilutions of plasma containing between 10^4 and 10^7 FIV molecules per ml as determined by quantitative competitive reverse transcriptase PCR (QC-PCR). No difference in clinical disease stage or mortality rate was associated with differences in FIV inoculum titers (8), so animals are hereafter referred to as a group rather than by virus input groups. For phase 4 experiments, 13-week-old specific-pathogen-free cats were infected by whole-blood passage from the phase 1 animal at 32 weeks p.i. This blood contained plasma with a virus titer of 10^4 molecules per ml (as determined by QC-PCR) as well as infected peripheral blood mononuclear cells.

QC-PCR. The QC-PCR assay for FIV in plasma was performed as previously described (7). Briefly, an RNA competitor was synthesized by PCR to amplify a DNA product containing a 21-bp internal deletion. This product was then cloned and used as a template for in vitro transcription of competitor RNA. Competitor RNA was quantified to determine RNA copy numbers and used to spike subsequent QC-PCR reactions. Plasma RNA was extracted with QIAamp Blood Kit reagents (Qiagen Inc., Chatsworth, Calif.) as per product directions. Sample RNA and 1 μl of serially diluted competitor RNA, containing from 10^2 to 10^9 copies of FIV RNA, were added to 96-well plates, and cDNA synthesis was performed with random primers and commercially available reagents (cDNA Cycle Kit; Invitrogen). Samples were then amplified by hot-start PCR and the following cycle program: 94°C for 10 s, 50°C for 15 s, and 72°C for 10 s for 30 cycles. Each reaction mixture contained 2 mM $MgCl_2$, 150 μM deoxynucleoside triphosphates, 8 U of AmpliTaq (Perkin-Elmer, Foster City, Calif.), and 0.5 μM each primer.

FIV antibody ELISA. Anti-FIV antibody in serum was measured as previously described (25). Briefly, virus from persistently FIV-infected CrFK cell superna-

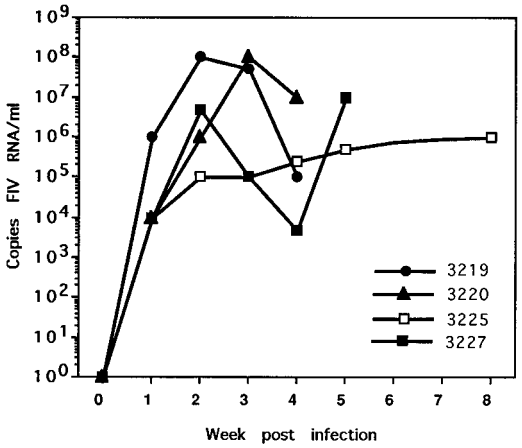

FIG. 1. Plasma virus kinetics in phase 2 cats. FIV RNA levels in plasma were determined by QC-PCR at weekly intervals throughout infection. Cats 3219 (circles) and 3220 (triangles) died at 4 weeks, cat 3227 (closed squares) died at 5 weeks, and cat 3225 (open squares) died at 8 weeks p.i.

tants was pelleted by ultracentrifugation, resuspended, purified by metrizamide-gradient centrifugation, and harvested, and the protein concentration was determined (Bio-Rad Laboratories, Richmond, Calif.). The virus was applied to 96-well microtiter plates at 500 μg per well in carbonate buffer, incubated for 2 h at room temperature, washed, and blocked with buffer containing 2% bovine serum albumin (BSA), 10% nonfat dry milk, and 5% donkey serum. Serum or plasma samples were then serially diluted in 100 μl of enzyme-linked immunosorbent assay (ELISA) buffer (TEN; 2% BSA, 4% fetal bovine serum, 0.5% Triton X-100) and incubated at room temperature for 2 h. The plates were washed with TEN containing 2% Tween 20 (polyoxyethylene sorbitan monolaurate). Peroxidase-labeled affinity-purified goat anti-feline immunoglobulin G (IgG) or IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted in buffer containing 5% horse serum, 5% donkey serum, and 5% mouse serum was used to detect bound serum antibody. TMB substrate (Kirkegaard & Perry Laboratories, Inc.) was used for the colorimetric reactions. Titers were expressed as the highest serum or plasma dilution which produced an optical density ≥10 times that of the negative control (naive specific-pathogen-free cat serum).

Western blot (immunoblot) analysis. The Western blot procedure was performed in a manner similar to that described previously (16, 36, 37). Supernatants from persistently FIV-infected CrFK cell cultures were clarified by centrifugation at 7,000 × *g* for 20 min and concentrated by Millipore Minitan ultrafiltration. Virus was then pelleted by ultracentrifugation at 36,000 rpm for 1 h, resuspended, and purified by metrizamide-gradient centrifugation. The viral protein concentrations were determined by Bio-Rad protein assay. The virus pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (0.015 mg of virus protein per gel), and viral proteins were separated by electrophoresis on a 12% polyacrylamide gel. The proteins were transferred to nitrocellulose, and the remaining binding sites were blocked with a 10% solution of nonfat milk powder in Western blot diluent and washing buffer (phosphate-buffered saline, Tween 80, EDTA, NaCl). The nitrocellulose was cut into strips, and each strip was incubated for 2 h with serum diluted 1:100 in Western blot diluent and washing buffer with 10% nonfat milk powder.

Each serum sample represents a single time point for an infected or control cat. After being washed repeatedly with Western blot diluent and washing buffers, bound serum antibody was detected with peroxidase-labeled goat anti-cat IgG polyclonal antibody and then with TMB substrate for development of the colorimetric reactions. Results are expressed as the percentages of either long-term-survivor or rapid-progressor cats having detectable serum antibodies to the Gag (10, 15, 24, 49, 52, or 55 kDa), Pol (32 or 62 kDa), transmembrane (42 kDa), and envelope glycoprotein (SU; 120 kDa) proteins.

RESULTS

Plasma virus load in rapid-progressor cats. Infection of 8-week-old cats with the FIV-C-PGammer acute-phase plasma pool resulted in marked clinical symptoms and 100% mortality by 8 weeks p.i. in phase 2 cats (8). Cats 3219 and 3227 were euthanized at 4 weeks p.i., cat 3220 was euthanized at 5 weeks p.i., and cat 3225 was euthanized at 8 weeks p.i. because of intractable illness. The severity of clinical disease and rapidity of progression in these cats were much greater than what has been reported for FIV (3, 9, 20, 21, 26, 38, 39). We have previously described viral kinetics for cats infected with the FIV Amelda 2542 isolate, a subgroup B virus, for which disease progression was less rapid (7). To determine if viral-load kinetics in the FIV-C-PGammer rapid-progressor cats were similar, FIV RNA was measured by QC-PCR at serial time points following infection in each animal (Fig. 1). Peak plasma RNA levels for three of four cats were between 10^7 and 10^8 copies per ml, which were comparable to the peak virus loads previously described (7). Initial viral RNA levels were lower, 10^5 copies per ml at 2 to 3 weeks p.i., in cat 3225 but increased steadily throughout infection.

Viral-load differences between rapid-progressor and long-term-survivor cats. In study phases 3 and 4, 12- and 13-week-old cats were infected with FIV-C-PGammer in an attempt to prolong the course of infection previously observed in 8-week-old cats. We observed that increased age at the time of infection attenuated the rapidity of disease progression in some animals (8). Over half of the phase 3 and 4 study animals developed rapidly fatal immunodeficiency disease, while others had moderate symptoms and then a prolonged asymptomatic period (8). We evaluated whether plasma virus load could predict the rate of disease progression in these animals.

In the phase 3 study, 7 of 12 FIV-infected 12-week-old cats developed intractable disease and were euthanized between 5 and 6 weeks p.i. These animals were designated rapid progressors, and average plasma virus kinetics data for this group are shown in Fig. 2. The remaining phase 3 study animals resolved their initial clinical symptoms by 6 to 7 weeks p.i. and remained asymptomatic throughout the remainder of the 38-week study. Average plasma FIV RNA load for this group is also shown (Fig. 2). The average plasma virus burdens in rapid-progressor animals were 1 to 2 logs higher than those of long-term survivors by week 2 and throughout infection.

To confirm these findings, we evaluated plasma virus kinetics in eight phase 4 cats of which five were rapid progressors and three recovered from early symptoms and remained asymptomatic for 29 weeks p.i. In this group, the rapid-progressor animals had plasma RNA levels an average of 1 to 2 logs higher than those of infected long-term survivors, and this difference could be seen by 1 week p.i. (Fig. 3). Plasma virus in long-term survivors declined after about 5 weeks p.i. but re-

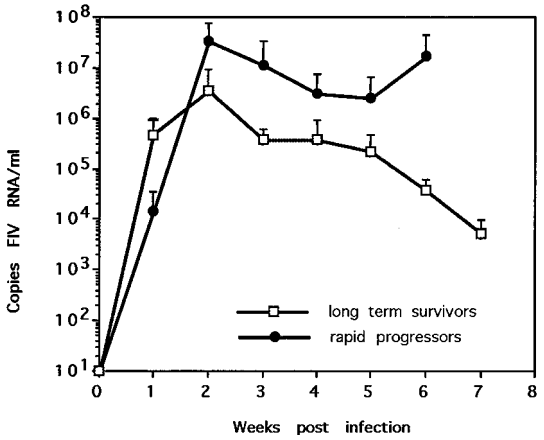

FIG. 2. Plasma FIV loads in 12-week-old phase 3 cats. Average weekly virus loads for rapid progressors (closed circles) and long-term survivors (open squares) were determined by QC-PCR at weekly intervals for the first 7 weeks p.i. Seven of 12 cats died within 6 weeks p.i. Long-term-survivor cats remained clinically asymptomatic through the remainder of the 38-week study.

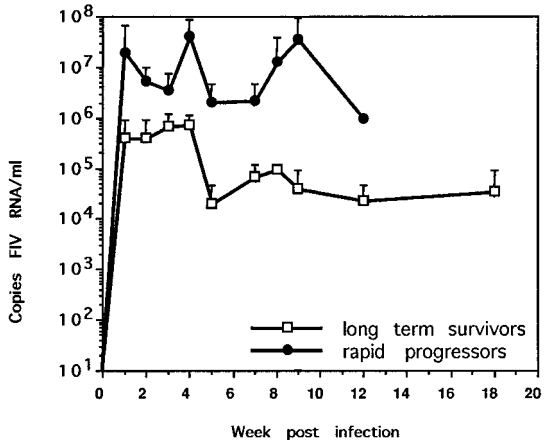

FIG. 3. Plasma virus kinetics in phase 4 cats. Five of 8 cats developed terminal disease within 12 weeks p.i. The average virus loads for these rapid-progressor cats (closed circles) and three long-term survivors (open squares) were determined by QC-PCR at weekly intervals throughout infection.

mained detectable throughout the study. We assessed the statistical significance of these findings using the Student *t* test to compare the plasma virus loads of 12 rapid progressors, from phases 3 and 4, to those of eight long-term survivors at each week p.i. *P* values were less than 0.05 at 2 weeks p.i. and thereafter, except at week 3 p.i., at which time the *P* value was 0.08, showing that the differences between rapid-progressor and long-term-survivor virus loads are statistically significant.

Anti-FIV antibody responses in rapid progressors and long-term survivors. Long-term survivors had significantly lower plasma FIV RNA levels than rapid progressors, indicating less systemic virus replication was occurring. We investigated whether antibody production could be associated with down regulation of virus replication and prolonged survival.

Serum antibodies recognizing FIV proteins were measured by a whole-virus ELISA. The results for phase 3 cats are shown in Table 1. Most cats developed a low, but detectable, IgM titer by 3 to 4 weeks p.i. One rapid progressor (cat 3264) and one long-term survivor (cat 3260) had no measurable FIV-specific IgM. All infected cats had IgG titers within the first 6 weeks of infection. Uninfected control animals (not shown) had no detectable IgM or IgG response. No differences in anti-FIV IgM or IgG levels were observed between rapid progressors and long-term survivors.

The ability to mount an antibody response against specific FIV proteins at serial time points following infection was also evaluated in phase 3 cats (Fig. 4). We found that while the Gag and transmembrane proteins were recognized by similar percentages of rapid progressors and survivors, the number of rapid-progressor animals capable of mounting an antibody response to the Pol protein and SU dropped precipitously in the terminal stages of infection. The decrease in anti-SU antibody production in rapid progressors was especially striking, as no response could be detected in the terminal-phase plasma of any rapid progressor while nearly 100% of long-term survivors had detectable SU antibodies.

DISCUSSION

Viral RNA load has been shown to indicate disease stage (28–31) and predict the rapidity of disease progression in HIV-1-infected individuals (14, 22, 33). We had previously demonstrated that plasma FIV RNA levels correlate with disease stage in infected cats (7). Here we expand upon those observations by demonstrating that plasma virus loads are significantly higher in cats which develop rapidly progressive disease following FIV-C-PGammer infection than in long-term survivors and that this higher virus load is a consistent feature throughout the course of infection. FIV replication kinetics reported in this study follow the pattern previously described (7), with an early peak viremia occurring within 1 to 3 weeks of infection followed by a decline and then a terminal increase in virus load. Early peak viral loads in FIV-C-PGammer-infected cats were comparable to those previously seen in cats infected with FIV subgroup B Amelda 2542 (7); however, virus loads remained high in rapid-progressor animals and returned to peak levels within 5 to 8 weeks. In FIV-C-PGammer long-term survivors and cats infected with FIV subgroup B Amelda 2542

TABLE 1. Anti-FIV antibody titers in 12-week-old phase 3 cats[a]

Week p.i.	Antibody titer[b] for cat:																							
	3257		3258		3261		3262		3264		3266		3270		3255		3260		3263		3267		3268	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	20	0	0	0	0	0	0	0	0	0	0	0	0	10	50	0	0	0	0	0	0	0	0	0
3	50	0	10	0	10	0	0	20	0	0	0	0	0	100	50	10	0	0	50	0	0	0	10	0
4	10	100	10	50	50	100	10	50	0	100	10	100	10	1,000	50	50	0	10	50	20	10	100	50	50
5	0	100	10	100	10	100	20	50	0	100	0	100	10	1,000	50	100	0	20	10	20	10	100	10	100
6	0	20	0	100	0	1,000	0	50	0	100	0	100	0	1,000	50	1,000	0	20	0	ND[c]	50	1,000	10	100
7																50		0		50		100		10
8																100		0		50		100		50
9																100		0		100		100		50
10																100		0		100		100		100
11																1,000		20		50		100		100
12																100		20		50		100		100
16																10,000		1,000		100		100		100
20																>10,000		>10,000		1,000		100		1,000

[a] Antibodies in sera recognizing FIV proteins were measured by a whole-virus ELISA. Cats with data through 6 weeks p.i. are rapid progressors; cats with data through 20 weeks p.i. are long-term survivors.

[b] Titers are expressed as the highest serum or plasma dilution that produced an optical density ≥10 times that of the negative control.

[c] ND, not determined.

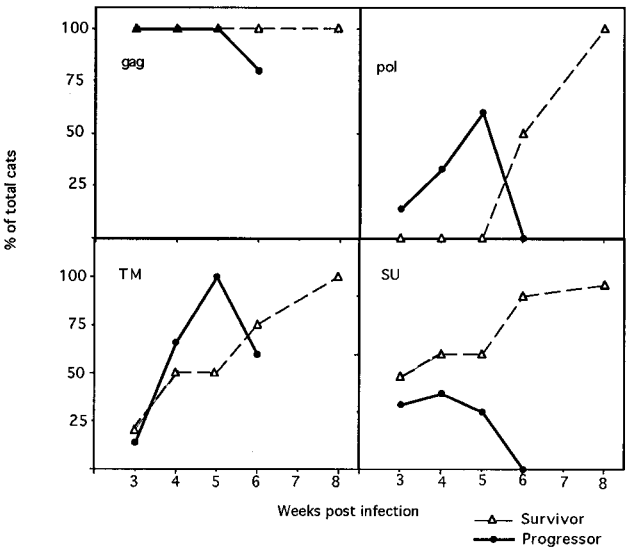

FIG. 4. Western blot analyses of antibody responses of survivor and rapid-progressor FIV-infected cats. The presence of antibodies recognizing FIV antigens in the serum samples of phase 3 survivor and rapid-progressor animals were compared. Results are expressed as the percentages of either long-term-survivor or rapid-progressor cats having detectable levels of antibody in sera to the Gag (10, 15, 24, 49, 52 or 55 kDa), Pol (32 or 62 kDa), transmembrane (TM; 42 kDa), and envelope glycoprotein (120 kDa) proteins.

(7), plasma virus loads declined and remained low for prolonged periods.

Both virus and host factors can affect virus replication rates and immunodeficiency disease progression in an individual (4, 5, 11). During development of the accelerated FIV disease model, cats were infected under conditions designed to provide the virus with an early replicative advantage (8). These conditions included the infection of cats with FIV in acute-phase plasma rather than with virus derived from tissue culture or from chronically infected, clinically asymptomatic animals. Thus, infection with high-titer FIV-C-PGammer resulted in acute-phase plasma producing rapidly fatal immunodeficiency disease in 100% of 8-week-old cats and ~60% of older animals. The results for 12-week-old or older cats demonstrated that identical virus inocula could result in either rapid or prolonged disease progression, indicating that host factors alone could account for differences in disease outcomes. That disease outcomes varied between same-sex littermates suggested that age, sex, and breed were not primary determinants.

Down regulation of virus load after the acute phase of lentivirus infection is thought to occur secondary to the host immune response (10, 32). An effective immune response might also result in a reduced acute-phase virus load and slowed disease progression. We evaluated humoral immune responses in infected cats to see if antibody responses correlated with survival times. While total anti-FIV antibody levels were similar between rapid-progressor and long-term-survivor animals, the percentage of rapid progressors capable of mounting an antibody response to Pol and SU proteins decreased dramatically in the terminal stages of infection. Determination of whether the diminished anti-Pol and -SU antibody production in rapid progressors predisposes the cats to or results from immunodeficiency will require additional investigation, as will the role of cellular immunity in FIV progression. Variation in individual immune responses is an attractive hypothesis for virus load differences; however, it is unclear whether the observed differences in antibody recognition patterns alone are significant since substantial differences in virus loads were apparent weeks before differences in antibody response patterns.

Here we show that viral RNA load is predictive of disease progression in FIV infection as it is in HIV-1 infection (22, 33). A similar phenomenon has been reported in the simian immunodeficiency virus model, in which monkey species resistant to immunodeficiency disease have little or no detectable circulating virus antigens compared with susceptible species, whose virus antigens are readily detectable (15). Likewise, antigenemia and the absence of detectable antibody responses have been correlated with rapid progression as opposed to the typical month's to year's duration of simian immunodeficiency virus infection (24). Preliminary results showing a correlation between virus load and disease outcome in simian immunodeficiency virus-infected macaques have also been reported (41).

One of the major applications of animal lentivirus infection models is to assess potential human therapies. We believe the parallels in virus loads and disease progressions between HIV-1 and FIV support the idea that the accelerated disease model is well suited for the study of therapeutic agents directed at reducing lentivirus replication.

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