Re-examination of feline leukemia virus: host relationships using real-time PCR

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Abstract

The mechanisms responsible for effective vs. ineffective viral containment are central to immunoprevention and therapies of retroviral infections. Feline leukemia virus (FeLV) infection is unique as a naturally occurring, diametric example of effective vs. ineffective retroviral containment by the host. We developed a sensitive quantitative real-time DNA PCR assay specific for exogenous FeLV to further explore the FeLV–host relationship. By assaying p27 capsid antigen in blood and FeLV DNA in blood and tissues of successfully vaccinated, unsuccessfully vaccinated, and unvaccinated pathogen-free cats, we defined four statistically separable classes of FeLV infection, provisionally designated as abortive, regressive, latent, and progressive. These host–virus relationships were established by 8 weeks post-challenge and could be maintained for years. Real-time PCR methods offer promise in gaining deeper insight into the mechanisms of FeLV infection and immunity.

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Introduction

Feline leukemia virus (FeLV) is a naturally occurring, contagiously transmitted, gammaretrovirus of cats (Hardy et al., 1973; Hoover et al., 1972; Jarrett et al., 1964; Kawakami et al., 1967; Rickard et al., 1969). Its pathogenic effects are paradoxical, causing both cytoproliferative (e.g., lymphoma or myeloproliferative disorder) and cytosuppressive (e.g., immunodeficiency or myelosuppression) disease (Anderson et al., 1971; Cockerell and Hoover, 1977; Cockerell et al., 1976; Hoover et al., 1974; Jarrett et al., 1964; Kawakami et al., 1967; Mackey et al., 1975; Perryman et al., 1972; Rickard et al., 1969). While many FeLV-exposed cats (estimated at ~30%) develop progressive infection and FeLV-related disease, at least twice as many (estimated at ~60%) develop regressive infection marked by an effective and durable immune response that contains and possibly extinguishes viral replication, thereby abrogating development of disease (Hardy et al., 1976; Hoover and Mullins, 1991; Hoover et al., 1981; Rojko et al., 1979). That effective host containment of FeLV infection can occur prompted research leading to development of the first vaccine for a naturally occurring retroviral infection (Hoover et al., 1991; Lewis et al., 1981; Sparkes, 1997).

Available evidence suggests that the interplay between the host and virus within the first 4 weeks after FeLV exposure results in either (a) failure of host immune response to contain viral replication in lymph nodes, epithelia, and bone marrow precursor cells or (b) successful host immune response resulting in curtailment of viral replication (Hoover and Mullins, 1991; Hoover et al., 1981; Rojko et al., 1979). Cats with progressive infection develop persistent antigenemia as detected by p27 capsid antigen capture in blood and have neither virus neutralizing antibodies (VN Ab) nor high levels of FeLV-specific cytotoxic lymphocytes (CTLs) (Flynn et al., 2000, 2002; Hoover and Mullins, 1991). By contrast, cats with regressive infection do not develop persistent antigenemia but do produce VN Ab and a detectable CTL response.
(Flynn et al., 2000, 2002; Hoover and Mullins, 1991). Because identification of FeLV infection has necessarily been based on assays that rely on viral replication and substantial viremia/antigenemia, it is unclear whether regressors retain latent (nonproductive) infection or instead may eliminate all cells bearing integrated FeLV provirus. Several laboratories have shown that it is possible to reactivate FeLV from some cats with regressive infection (Madewell and Jarrett, 1983; Post and Warren, 1980; Rojko et al., 1982). Despite this, attempts by other laboratories to amplify viral DNA sequences in circulating and/or bone marrow cells from cats with suspected latent infections have been unsuccessful using conventional PCR (Herring et al., 2001; Jackson et al., 1996; Miyazawa and Jarrett, 1997). Similar to FeLV regressors, protected vaccinates do not develop persistent antigenemia. To the authors knowledge, however, studies assessing vaccinates for potential latent infections using PCR have not been performed (Sparkes, 1997).

Recent studies employing quantitative real-time PCR in experimental FeLV infections have shown that the early circulating proviral burden influences the course of infection and that real-time PCR detected provirus in circulating cells from cats with undetectable or transient antigenemia (Flynn et al., 2002; Hofmann-Lehmann et al., 2001). To explore further the FeLV–host relationship and assess the presence of latent viral DNA in circulation and tissue, we developed a quantitative real-time PCR assay and examined the early (weeks post-challenge) and late (years post-challenge) phases of experimental FeLV infection in both unvaccinated animals and those primed by vaccination. Here we examine proviral and p27 levels in FeLV-61E-A-challenged cats given effective, ineffective, or no FeLV vaccine. Based on the results of these studies, we suggest four categories within the spectrum of FeLV infection, which we have provisionally designated as abortive, regressive, latent, and progressive.

**Results**

**Validation of FeLV quantitative real-time PCR**

**Specificity**

The analytical specificity of the FeLV quantitative real-time PCR assay was confirmed by sequencing two amplicons after agarose gel confirmation (data not shown). Using BLAST (Altschul et al., 1990; Wheeler et al., 2003), the amplicon sequences were shown to be identical to that of FeLV-61E-A (data not shown). Peripheral blood mononuclear cells (PBMC) and lymphoid tissues from FeLV-naive, specific-pathogen-free (SPF) cats were consistently negative for FeLV DNA (49/49 samples from 18 cats; data not shown); thus, endogenous FeLV sequences were not amplified. Consequently, diagnostic specificity was 100% in FeLV-61E-A-infected animals.

**Sensitivity**

The analytical sensitivity of the FeLV real-time PCR assay was assessed in end-point dilution experiments. These studies consistently detected five copies of the p61E-FeLV plasmid standard (data not shown). The template control (no DNA, PCR-grade H$_2$O only), negative control (FeLV-naive, SPF cat DNA), and samples containing 0.5 copy of the plasmid standard never crossed threshold. All FeLV-61E-A-infected cats that tested positive for p27 capsid antigen also were positive by real-time PCR (76/76 samples from 23 cats) (Table 1). Thus, diagnostic sensitivity in the animals studied was 100%.

**Linearity**

The linear range of the plasmid standard curve was evaluated. Amplification of 10-fold serial dilutions starting at $5 \times 10^3$ copies and ending at $5 \times 10^9$ copies of the p61E-FeLV plasmid standard from 18 independent experiments demonstrated linearity over 8 orders of magnitude, generated a standard curve correlation coefficient of 0.999, and produced an amplification efficiency (Klein et al., 2001) of 96.6% (data not shown).

**Amplification efficiency**

The amplification efficiencies of FeLV-61E-A-infected cat DNA and the p61E-FeLV plasmid standard were compared to validate quantification using the plasmid standard. Equivalent amplification efficiencies are indicated by regression line slopes ($s$) with less than 0.1 difference ($\Delta s$) (Gut et al., 1999). The observed amplification efficiencies of the target DNA ($s = 3.32$, $R^2 = 0.997$) vs. the plasmid standard ($s = 3.30$, $R^2 = 0.999$) had a $\Delta s = 0.02$ (data not shown). Thus, quantification using the plasmid standard was expected to be valid.

**Reproducibility**

The within-run and between-run precision of the FeLV real-time PCR assay was evaluated. Several dilutions of the p61E-FeLV plasmid standard and of FeLV-61E-A-infected cat DNA were amplified 10 times within the same reaction plate and between 10 different reaction plates. The threshold cycle coefficients of variation, $CV(C_T)$, for the within-run precision was 0.31–1.11% and the $CV(C_T)$ for the between-run precision was 0.56–1.16% (data not shown). Thus, the assay was considered highly reproducible.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Real-time PCR (+)</th>
<th>Real-time PCR (−)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p27 ELISA</td>
<td>(+) 76</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>(−) 24</td>
<td>23</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>23</td>
<td>123</td>
</tr>
</tbody>
</table>

Kappa value = 0.53 (fair agreement).
Early circulating p27 and viral DNA levels in FeLV-challenged animals

Sera and PBMC collected pre-challenge and every 2 weeks thereafter through 8 weeks post-challenge (PC) were analyzed for FeLV p27 capsid antigen via capture ELISA and for FeLV U3 LTR DNA via quantitative real-time PCR. None of the cats had detectable antigen or viral DNA pre-challenge.

Animals receiving Vaccine A

FeLV p27 was never detected in 9 of the 10 cats (90%), which received Vaccine A (Fig. 1A). Of these nine protected vaccinates, four never had detectable viral DNA, two developed transient low provirus loads (median: 130 copies/10^6 PBMC; range: 0 to 1723 copies/10^6 PBMC), which gave way to undetectable levels (1 cat by 6 weeks and 1 cat by 8 weeks), and three had persistent low viral DNA levels (median: 225 copies/10^6 PBMC; range: 0 to 7744 copies/10^6 PBMC) (Fig. 1B). In the one persistently antigenemic failed vaccinate, a persistent high proviral burden was present PC (median: 477,999 copies/10^6 PBMC; range: 17,140 to 578,572 copies/10^6 PBMC).

Animals receiving Vaccine B

Thirteen of the 15 cats (86%) given Vaccine B developed persistent antigenemia and persistent high proviral burdens PC (median: 259,013 copies/10^6 PBMC; range: 7330 to 2,224,869 copies/10^6 PBMC). p27 was never detected in the remaining two vaccinates. In one of these latter animals, viral DNA also was never detected whereas in the second animal, persistent low proviral load (median: 18,790 copies/10^6 PBMC; range: 6079 to 30,854 copies/10^6 PBMC) was present.

Unvaccinated controls

Seven of the 10 unvaccinated control cats (70%) developed persistent antigenemia and high proviral burdens PC (median: 265,572 copies/10^6 PBMC; range: 11,942 to 1,508,006 copies/10^6 PBMC). The remaining three animals experienced transient antigenemia between 2 and 6 weeks PC, after which p27 was no longer detectable (1 cat by 4 weeks and 2 cats by 6 weeks). These latter cats retained persistent moderate proviral burdens (median: 40,969 copies/10^6 PBMC; range: 860–328,249 copies/10^6 PBMC).

Using repeated measures-ANOVA and the Tukey–Kramer post hoc test, statistically significant differences (P < 0.01) in p27 and viral DNA levels were present between Vaccine A vs. Vaccine B and between Vaccine A vs. unvaccinated Controls. Results for Vaccine B were not statistically different from the unvaccinated Controls.

Preventable fraction

The preventable fraction (PF) is used to express vaccine efficacy due to the inherent resistance of approximately 60% of unvaccinated cats to development of persistent antigenemia after FeLV challenge (Pollack and Scarlett, 1990), as shown in Eq. (1). The PF for Vaccine A was 85.7%. The PF for Vaccine B was −23.8%.

\[
PF = \frac{\text{Incidence of Persistent Antigenemia in Controls} - \text{Incidence of Persistent Antigenemia in Vaccinates}}{\text{Incidence of Persistent Antigenemia in Controls}}
\] (1)

Host–virus relationships defined using circulating p27 and viral DNA levels

In the original FeLV–host relationship classification scheme, FeLV-exposed animals that did not develop persistent antigenemia were identified as having experienced regressive infections. The results of the present study suggest that FeLV-exposed antigen-negative cats represent a spectrum of host–virus relationships.

The five FeLV-61E-A-inoculated cats in which neither p27 nor viral DNA were detected at any time were classified as having experienced abortive infection (Table 2; Fig. 2). Four of these cats were vaccinated with Vaccine A and 1 with Vaccine B.

The six cats that never developed detectable antigenemia but in which transient or low persistent circulating viral DNA levels were detectable (median: 225 copies/10^6 PBMC; range: 0–30,854 copies/10^6 PBMC) were classified as having experienced regressive infection. Five of these cats were vaccinated with Vaccine A and 1 with Vaccine B. An initial low proviral burden detected at 4 weeks PC was no longer demonstrable by 8 weeks PC in two cats vaccinated with Vaccine A.

Transient antigenemia was demonstrated in three unvaccinated control cats that retained persistent moderate proviral loads in blood (median: 40,969 copies/10^6 PBMC; range: 860–328,249 copies/10^6 PBMC). These animals were classified as retaining latent infection.

Finally, 21 cats developed persistent antigenemia with concurrent persistent high circulating proviral burdens (median: 269,328 copies/10^6 PBMC; range: 7330–2,224,869 copies/10^6 PBMC). These animals, as in previous classification schemes, were designated as progressive infection.

Using repeated measures-ANOVA and the Tukey–Kramer post hoc test, statistically significant differences (P < 0.01) in p27 values were identified between progressive vs. abortive, progressive vs. regressive, and progressive vs. latent infection. Statistically significant differences (P < 0.01) in viral DNA burdens were present among all FeLV categories (with the exception of latent vs. progressive infection): abortive vs. regressive, abortive vs. latent, abortive vs. progressive, regressive vs. latent, and regressive vs. progressive infection.
Agreement and correlation between p27 and viral DNA detection

The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the p27 capture ELISA and the real-time PCR assay (Table 1). All samples that tested positive for p27 capsid antigen were positive by real-time PCR (76 samples from 23 cats). All samples with undetectable viral DNA (real-time PCR negative) had undetectable antigen (ELISA negative) (23 samples from 8 cats). No sample was positive by ELISA and negative by real-time PCR. However, 24 samples from 13 cats were positive by real-time PCR and negative by p27 capture. Thus, real-time PCR had greater

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Response category</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abortive</td>
<td>Regressive</td>
</tr>
<tr>
<td>Provirus (-)</td>
<td>Provirus (+)²</td>
<td>Provirus (++)</td>
</tr>
<tr>
<td>Antigen (-)</td>
<td>Antigen (-)</td>
<td>Antigen (+)²(-)</td>
</tr>
<tr>
<td>Vaccine A</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Vaccine B</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

² After detecting an initial low proviral load, two of the six cats with regressive infection did not have detectable provirus at 8 weeks post-challenge. Both cats received Vaccine A.
sensitivity than p27 capture ELISA. The kappa statistic was 0.53, indicating a fair agreement between the two tests.

Pearson correlation coefficients were determined to assess the linear relationship between circulating p27 levels and PBMC viral DNA levels. After a Fisher's \( r \to z \) transformation, \( P \) values were obtained. The correlation between ELISA and real-time PCR became progressively more concordant as infections became fully established as indicated by the following trend in time periods: 2 weeks PC \( r = 0.761, P < 0.01 \); 4 weeks PC \( r = 0.461, P < 0.05 \); 6 weeks PC \( r = 0.555, P < 0.01 \); and 8 weeks PC \( r = 0.640, P < 0.01 \). After splitting the data by category of FeLV infection, a more linear relationship between the assays appeared: abortive infection \( r = \) not applicable (no variability in the data); regressive infection \( r = 0.831, P < 0.01 \); latent infection \( r = 0.896, P < 0.01 \); and progressive infection \( r = 0.409, P < 0.01 \).

Long-term outcome and host–virus relationships in 13 of the FeLV-challenged cats

Thirteen of the 35 cats studied above were available for necropsy after survival periods of 2–3.5 years. This cohort was comprised of five cats from the Vaccine A group, four cats from the Vaccine B group, and four from the unvaccinated Control group (Table 3). Sera were analyzed for p27 capsid antigen via capture ELISA. PBMC, bone marrow (BM), spleen (SP), and mesenteric lymph node...
from all 13 animals were analyzed for viral DNA via quantitative real-time PCR. In addition, thymus, tonsil, and retropharyngeal lymph node were available for the five cats vaccinated with Vaccine A.

Abortive infection

Three cats that received Vaccine A and were categorized as abortive infection (antigen negative/provirus negative) remained antigen and provirus negative in blood after a 2-year observation period (Fig. 3). Perhaps surprisingly, viral DNA was not detectable in the BM, SP, or MLN of these same animals. In addition, no viral DNA could be detected in thymus, tonsil, or retropharyngeal lymph node (data not shown). It would not be possible, therefore, to distinguish these animals from those never exposed to FeLV on the basis of antigen capture ELISA and viral DNA real-time PCR assay results alone.

Regressive infection

Two cats that received Vaccine A and were classified as regressive infection (antigen negative/low transient provirus) (Table 2) also remained antigen- and provirus-negative in blood nearly 2 years later. Similar to cats with abortive infections, viral DNA was not detected in BM, SP, or MLN, nor was it detected in thymus, tonsil, and retropharyngeal lymph node (data not shown). The one cat that received Vaccine B and was classified as regressive infection (antigen negative/persistent low proviral load) remained antigen negative without detectable viral DNA in PBMC, BM, SP, and MLN. The one unvaccinated control cat classified as latent infection became p27 positive with detectable viral DNA in PBMC, BM, SP, and MLN. The three Vaccine B cats and three unvaccinated control cats with progressive infection remained p27 positive with readily detectable viral DNA in PBMC, BM, SP, and MLN. Pearson correlation coefficients and P values between PBMC and tissues were PBMC vs. BM: r = 0.559, P < 0.05; PBMC vs. SP: r = 0.975, P < 0.01; and PBMC vs. MLN: r = 0.823, P < 0.01. Means ± SD are plotted. Category of FeLV infection as classified by the p27 and viral DNA assays during the first 8 weeks post-challenge. A = abortive, R = regressive, L = latent, and P = progressive.

Experimental group. VA = vaccine A, VB = vaccine B, C = unvaccinated control. VA represents results from 3 cats and VB from 2 cats, whereby neither circulating nor tissue viral DNA was detected at euthanasia.
negative. The relatively low PBMC viral DNA levels detected at 8 weeks PC (6866 ± 668 copies/10⁶ PBMC) were retained 3 years later (44 ± 76 copies/10⁶ PBMC) and these levels were similar to those detected in BM, SP, and MLN.

**Latent infection**

The one unvaccinated control cat classified as latent infection (transient antigenemia/persistent moderate proviral load) had become p27-positive 3 years later. Viral DNA levels detected in PBMC of this animal were similar to BM, SP, and MLN although PBMC levels (919 ± 330 copies/10⁶ PBMC) after 3 years were appreciably lower than those detected at 8 weeks PC (94,184 ± 4962 copies/10⁶ PBMC).

**Progressive infection**

One cat that received Vaccine B and was considered to have progressive infection also remained unchanged 3 years later. The PBMC proviral load in this animal peaked at 4 weeks PC (518,096 ± 17,778 copies/10⁶ PBMC), decreased by 8 weeks PC (7330 ± 133 copies/10⁶ PBMC), and remained relatively similar to the 8-week level 3 years later (196 ± 63 copies/10⁶ PBMC). Proviral burdens in BM, SP, and MLN were similar to blood levels. Two cats that received Vaccine B and three unvaccinated control cats that were classified as progressive infections (antigen positive/persistent high proviral load) remained antigen-positive. The relatively high PBMC viral DNA levels detected at 8 weeks PC (639,174 ± 593,815 copies/10⁶ PBMC) were retained 3–3.5 years later (2,143,280 ± 1,387,100 copies/10⁶ PBMC) and these levels were similar to those detected in BM, SP, and MLN. Viral DNA levels in circulating cells correlated with levels in tissues. Pearson correlation coefficients between circulating and tissue viral DNA levels and the levels in tissues. Pearson correlation coefficients between PBMC and these levels were similar to those detected in BM, SP, and MLN.

**Discussion**

The primary purpose of this study was to develop and validate a quantitative real-time DNA PCR assay to examine FeLV-vaccinated and unvaccinated cats for viral DNA sequences in circulating cells during the early phase of FeLV infection and both circulating cells and tissue during the late phase of FeLV infection. This assay was based on an FeLV U3 LTR sequence and proved to be reproducible, quantitative, sensitive, and specific for exogenous FeLV. The greater sensitivity of real-time PCR allowed detection of viral DNA in cats with undetectable antigenemia. This finding is consistent with recent studies of Flynn et al. (2002) and Hofmann-Lehmann et al. (2001). The current real-time PCR assay, while similar to that developed by Hofmann-Lehmann et al. (2001), is based on FeLV-61E-A, the highly replication competent, non-acutely pathogenic component of the FeLV–FAIDS complex (Donahue et al., 1988; Hoover et al., 1987; Mullins et al., 1986; Overbaugh et al., 1988). The U3 LTR region is conserved among FeLV subgroup A viruses; thus, it is probable that detection of cross-isolates will occur using the present primer/probe set, although this issue was not addressed in the present study. While unintegrated viral DNA (UVD) is a characteristic of the FeLV-FAIDS strain, this method cannot distinguish between integrated provirus and UVD.

This would appear to be the first study assessing the efficacy of an FeLV vaccine using real-time PCR. Nine of the 10 cats that received Vaccine A (Fort Dodge Fel-O-Vax Lv-K) were protected as indicated by the absence of circulating FeLV p27. Moreover, in four of the nine protected vaccinates viral DNA was never detected in PBMC. The remaining five protected cats had either transient low (two cats) or persistent low (three cats) circulating viral DNA levels within the first 8 weeks PC. Importantly, viral DNA was not detectable in PBMC or lymphoid tissues from the five available animals, nearly 2 years after viral challenge. Previous studies examining the efficacy of Fel-O-Vax Lv-K reported preventable fractions of 86% and 100% (Hoover et al., 1995, 1996; Legendre et al., 1991). Virus was not isolated from bone marrow cultures at 7 or 31 weeks post-challenge/exposure in these experiments (Hoover et al., 1995, 1996; Legendre et al., 1991). Results of the present study bolster these previous findings, as do those of Hafler et al. (1987) lending support to the tenet that successful immunity to retroviral infection can be obtained with immunoprophylaxis.

The greater sensitivity of real-time PCR allowed us to suggest more detailed FeLV–host relationship categories, which we designated as: abortive, regressive, latent, and progressive. Although it is certainly plausible that these categories of FeLV infection may be dynamic, especially the intermediate categories, we found these host–virus relationships became established by 8 weeks and were maintained for 2–3.5 years in blood and lymphoid tissues.
et al., 1996; Miyazawa and Jarrett, 1997). Thus, it was proposed that these antigen-negative cats did not harbor latent virus in the sites examined. The results of the present study suggest that neither scenario is absolute. Rather, FeLV-exposed antigen-negative cats represent a spectrum of host–virus relationships wherein some animals appear to eliminate infected cells in circulation and tissues while some maintain a low to moderate level of infected cells. Reactivation is possible in the latter animals.

We hypothesize that cats with abortive infection produced effective early host immune responses, which abrogate viral replication and eliminate FeLV-infected cells. This is inconsistent with the hypothesis that all FeLV-exposed antigen-negative cats harbor a reservoir of infected cells in some hemolymphatic tissue. It remains possible, though not probable in our view, that such animals harbor sequestered FeLV in tissues not examined. It is also possible that the real-time PCR assay is not sufficiently sensitive to detect extraordinarily low proviral levels, as has been proposed to occur in people who are repeatedly exposed to human immunodeficiency virus yet remain seronegative (Zhu et al., 2003). Our present observations bolster the contention that some individuals can resist retroviral infection without conventional evidence of infection.

We propose that cats with regressive infection successfully contain viral replication despite retaining a low level of FeLV-infected cells in circulation and tissues. Some of these animals even eliminate these infected cells and go on to resemble cats with abortive infections. This supports the hypothesis that some FeLV-exposed antigen-negative cats can maintain populations of nonproductive, infected cells. Our results also demonstrate that these cats harbor viral DNA in circulation and lymphoid tissues in addition to bone marrow. While reactivation of regressive infection may be possible, this was not detected in the present study. Overall, the present study suggests a more likely outcome of eventual elimination or extinction of infected cells.

We propose in cats classified as latent infection that delayed containment of viral replication occurs resulting in a moderate proviral residuum. As a corollary, if host immune containment wanes, viral reactivation becomes more likely. This is consistent with the tenet that some FeLV-exposed antigen-negative cats can maintain cell populations harboring replication-competent latent FeLV capable of reactivation.

We assume that residual viral DNA detected by real-time PCR could represent intact provirus or replication-defective sequences. Previous studies have reported that nonviremic cats from which FeLV was isolated from cultured BM cells did not horizontally transmit FeLV (Madewell and Jarrett, 1983; Pacitti and Jarrett, 1985; Pedersen et al., 1984). However, vertical transmission to offspring from similar animals also has been reported (Pacitti et al., 1986; Pedersen et al., 1984). Additional studies are needed to assess the state and fate of viral DNA in latently infected cats. Such issues are pertinent to use of FeLV antigen-negative cats for blood donation, tissue transplants, and adoptions, as well as to the use of therapeutic immunosuppressive drugs in antigen-negative cats (Coronado et al., 2000; Gregory et al., 1991; Nemzek et al., 1994, 1996).

That effective containment of human immunodeficiency virus may be possible is inferred by long-term nonprogression in HIV-infected individuals and apparent resistance to infection in highly HIV-exposed seronegative individuals. Genetic, virological, and immunological factors all likely play a role in HIV containment (Cohen et al., 1997; Haynes et al., 1996; Levy, 1993; Rowland-Jones and McMichael, 1995). Animal models present unique opportunities to prospectively examine the initial events in immunopathogenesis. Further examination of the early immune responses that determine effective vs. ineffective containment of FeLV infection and better characterization of the latent viral state would provide valuable insights into retroviral pathogenesis and resistance overall.

Materials and methods

Study design

This is a retrospective analysis. Samples were utilized from two previous vaccine experiments. Experiment 1 consisted of five groups: group 1 received Vaccine A, groups 2, 3, and 4 all received Vaccine B but each by different routes of administration, and group 5 served as the Control as these cats did not receive any vaccination. Using repeated-measures ANOVA, no statistically significant differences were detected between the three groups that received Vaccine B by different routes of administration ($P = 0.47$, power = 0.15). Consequently, results from the three groups that received Vaccine B were combined. Experiment 2 consisted of two groups: group 1 received Vaccine A and group 2 served as the Control as these cats did not receive any vaccination. Again, no statistically significant differences were detected between the Vaccine A groups from Experiment 1 and 2 ($P = 0.16$ power = 0.27) or between the Control groups from Experiment 1 and 2 ($P = 0.53$, power = 0.09). Thus, results from the Vaccine A groups from Experiment 1 and Experiment 2 were combined and results from the Control groups from Experiment 1 and Experiment 2 were combined. In summary, this study presents the results from a combined total of 3 groups: Vaccine A, Vaccine B, and Control (Table 3).

Experimental animals

Thirty-five specific-pathogen-free (SPF) cats were obtained from Cedar River Laboratories (Mason City, IA) and randomly divided into seven groups, each group consisting of five cats (Table 3). Each group was individually housed at Laboratory Animal Resources at Colorado State University (Fort Collins, CO) in accordance with the
Vaccination

Ten cats were administered Vaccine A, the commercial FeLV vaccine Fel-O-Vax Lv-K (Fort Dodge Animal Health, Overland Park, KS) (Hoover et al., 1995, 1996), according to the manufacturer’s specifications (Table 3). Five cats received the subcutaneous priming vaccination at 15–16 weeks of age and a subcutaneous boosting vaccination at 19–20 weeks of age. The other five cats received the prime at 25–27 weeks of age and the boost at 31–33 weeks of age. Fifteen cats were administered Vaccine B, an experimental whole inactivated FeLV-Sarma-A with monophosphoryl lipid A adjuvant (MPL) (Corixa Corp., Seattle, WA), by different routes of administration. All 15 cats received the priming vaccination at 15–16 weeks of age and a boosting vaccination at 19–20 week of age. Five cats received an intranasal prime and boost, five cats received a subcutaneous prime and an intranasal boost, and five cats received a subcutaneous prime and no boost. Ten cats did not receive any vaccinations and served as the Controls.

Virus challenge

All cats were challenged oronasally with 1 mL of 10^4 TCID/mL FeLV-61E-A via dropwise instillation of 0.25 mL in each nostril and 0.5 mL in the mouth. This subgroup A virus strain is the highly replication competent, non-acutely pathogenic component of the FeLV–FAIDS complex (Donahue et al., 1988; Hoover et al., 1987; Mullins et al., 1986; Overbaugh et al., 1988). The cell-free infectious virus inoculum was prepared as supernatant from Crandell feline kidney (CrFK) cell cultures and determined to be equivalent to 1 CID_{100} (100% cat infective dose). The vaccines were challenged 3 weeks after receiving their boosting immunization; either 22–23 or 34–36 weeks of age (Table 3). Five control cats were challenged at 22–23 weeks of age and the other five at 34–36 weeks of age. All cats were observed daily for signs of illness after virus inoculation.

Sample collection and processing

Blood samples were collected at challenge and every 2 weeks thereafter through 8 weeks post-challenge (PC). Sera were stored at −20°C until analysis for FeLV p27 capsid antigen by capture ELISA. Peripheral blood mononuclear cells (PBMC) were isolated from blood by ficoll-hypaque (Histopaque-1077; Sigma Diagnostics, St. Louis, MO) density gradient centrifugation, separated into 1 × 10^6 PBMC/mL aliquots, and stored at −80°C until analysis by FeLV quantitative real-time PCR. DNA was extracted from PBMC using a QIAamp DNA blood mini kit (Qiagen, Inc., Valencia, CA), eluted in 100 μL of elution buffer, and DNA concentrations determined spectrophotometrically.

Detection of circulating p27 capsid antigen by capture ELISA

FeLV p27 capsid antigen was detected in serum by capture ELISA using the monoclonal antibodies (mAbs) anti-p27 A2 and G3 (Lutz et al., 1983) (kindly provided by Niels C. Pedersen; University of California, Davis, CA) as previously described (Zeidner et al., 1990) with minor adaptations. Briefly, 0.5 μg/well of the primary mAb, G3, was used to coat a 96-well plate, 50 μL of control or sample sera was added in duplicate to plate wells, and 50 μL of the secondary horseradish peroxidase-conjugated mAb, A2 at 1:250, was added and incubated for 45 min. The plates were then rinsed and 100 μL/well TMB peroxidase substrate/peroxidase solution B (H_2O_2) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added for color development. After a 15-min incubation, reactions were stopped with 50 μL/well 2N H_2SO_4 and optical density measurements were taken at 450 nm. Background readings, using FeLV-naive SPF cat serum, were subtracted from each well. Sample well reactions were considered positive if an absorbance value of 10% or more of the positive control (persistent antigenemic FeLV-infected cat serum) was obtained.

Detection and quantification of circulating and tissue FeLV viral DNA by quantitative real-time PCR

Using Primer Express software (Applied Biosystems, Foster City, CA), we designed a primer/probe set within the U3 region of the FeLV-61E-A long terminal repeat (LTR) (GenBank accession number M18247) (Donahue et al., 1988), thereby amplifying the exogenous but not endogenous FeLV sequences (Berry et al., 1988; Casey et al., 1981). The forward, 5’ AGTTCGACCTTCCGCTCAT 3’ (20 bases; nt 241–260), and reverse, 5’ AGAAAGC-
GGCGGTACAGAAG 3' (20 bases; nt 308–289), primer sequences amplified a 68-bp fragment. The corresponding probe sequence, 5' TAAACTACCAATCCCCTATG-CCTCTGC 3' (28 bases; nt 262–289), was labeled with the reporter dye, FAM (6-carboxyfluorescein), at the 5' end and the quencher dye, TAMRA (6-carboxytetramethylrhodamine; Applied Biosystems) or BHQ-1 (Black Hole Quencher-1; Biosource International, Inc., Camarillo, CA), at the 3' end. Both probes were blocked at the 3' end to prevent extension. The two probes produced similar results.

The 25-μL reaction consisted of 400 nM of each primer, 80 nM of fluorogenic probe, 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 3.5 μL of PCR-grade H2O, and 5 μL of sample or plasmid standard DNA. The master mix was supplied at a 2× concentration and contained AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTPs with dUTP, and optimized buffer components. Reactions were performed in triplicate using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Every reaction plate contained a template control (no DNA, PCR-optimized buffer components). Reactions were performed to allow enzymatic activity of UNG, 10 min at 95 °C to reduce UNG activity, to activate AmpliTaq Gold DNA Polymerase, and to denature the template DNA, followed by 40 cycles of 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing/extension.

The plasmid p61E-FeLV, an EcoRI fragment containing the full-length FeLV-61E-A provirus subcloned into pUC18 (Donahue et al., 1988; Overbaugh et al., 1988), was used as the standard for PCR quantification. The plasmid was provided as ampicillin-resistant transformed E. coli JM109 cells through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. James Mullins. The transformed E. coli cells were grown on LB media containing 50 μg/mL ampicillin. Plasmid DNA was isolated from the bacterial cells using the QIAfilter plasmid midi kit (Qiagen, Inc.), linearized with EcoRI, and the plasmid insert confirmed by agarose gel electrophoresis with GelStar (BioWhittaker Molecular Applications) staining. Two cloned inserts were sequenced by Davis Sequencing LLC (Davis, CA). The sequences of the PCR products were then aligned with FeLV-61E-A using BLAST (Altschul et al., 1990; Wheeler et al., 2003) on the National Center for Biotechnology Information website.

End-point dilution experiments of the p61E-FeLV plasmid standard were performed to assess analytical sensitivity. A dilution series of 500, 100, 50, 10, 5, 1, 0.5, and 0.1 copies of the plasmid standard, each in triplicate, was tested.

Amplification efficiency and reproducibility of FeLV quantitative real-time PCR

To assess amplification efficiencies, serial dilutions (1:10, 1:100, 1:1000, and 1:10000) of PBMC DNA from an experimentally FeLV-61E-A-infected cat and of the p61E-FeLV plasmid standard were amplified in triplicate and the difference in the slopes (ΔΔ) of the regression lines (C_T vs. dilution) was evaluated.

To assess assay reproducibility, dilutions of the p61E-FeLV plasmid standard (50000, 5000, and 500 copies) and of DNA from an experimentally FeLV-61E-A-infected cat (100%, 1:100, and 1:1000) were evaluated for within-run and between-run precision. Each dilution was run 10 times within the same reaction plate and between 10 different reaction plates to test the within-run and between-run precision, respectively. The coefficients of variations (CV) of the threshold cycles (C_T) were calculated: CV (C_T).

Statistics

Statistically significant differences in p27 and viral DNA levels (log transformed) between the experimental groups and between the FeLV–host categories were determined using repeated-measure analysis of variance (ANOVA) with the Tukey–Kramer post hoc test. A statistically significant difference between groups was considered to have occurred when a P value was <0.05. The kappa statistic was calculated to assess the level of agreement, beyond that which might be expected due to chance, between the p27

Analytical specificity and sensitivity of FeLV quantitative real-time PCR

Following agarose gel electrophoresis confirmation with GelStar (BioWhittaker Molecular Applications, Rockland, ME) staining, the 68-bp PCR products from two separate reactions were sequenced to verify analytical specificity. The TOPO TA Cloning Kit (with pCR 2.1-TOPO vector) (Invitrogen Corp., Carlsbad, CA) was used for cloning the amplicons prior to sequencing. Briefly, the PCR products were directly ligated into the linearized pCR 2.1-TOPO vector (Invitrogen Corp.), the constructs were transformed into One Shot TOP 10 chemically competent E. coli cells (Invitrogen Corp.), and the cells grown on LB media with 50 μg/mL ampicillin using blue/white screening. Plasmid DNA was isolated from the bacterial cells using the QIAfilter plasmid midi kit (Qiagen, Inc.), linearized with EcoRI, and the plasmid insert confirmed by agarose gel electrophoresis with GelStar (BioWhittaker Molecular Applications) staining. Two cloned inserts were sequenced by Davis Sequencing LLC (Davis, CA). The sequences of the PCR products were then aligned with FeLV-61E-A using BLAST (Altschul et al., 1990; Wheeler et al., 2003) on the National Center for Biotechnology Information website.
capture ELISA and the real-time PCR assay. Pearson correlation coefficients were determined to assess the linear relationship between circulating p27 levels vs. PBMC viral DNA levels and between circulating vs. tissue viral DNA levels. After a Fisher’s r to z transformation, P values were obtained. Again, a statistically significant difference between groups was considered to have occurred when the P value was <0.05. Repeated-measures ANOVA, the Tukey–Kramer post hoc test, and the Pearson correlation coefficient were performed using StatView version 5.0.1 for Macintosh, copyright 1999 (Abacus Concepts, Inc., Berkeley, CA).

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