Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats

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Feline leukaemia virus (FeLV) infection in domestic cats can vary in its outcome (persistent, transient, no infection) for reasons that are not entirely known. It was hypothesized that the initial virus and provirus load could significantly influence the course of retrovirus infection. To determine the role of provirus loads, two methods of PCR, a nested PCR and a fluorogenic probe-based (TaqMan) real-time quantitative PCR, which were specific to the U3 region of FeLV-A were established. FeLV provirus in naturally and experimentally infected cats was then measured. Only 3 weeks after experimental FeLV-A infection, persistently infected cats demonstrated higher provirus loads and lower humoral immune responses than cats that had overcome antigenaemia. Lower initial provirus loads were associated with successful humoral immune responses. Unexpectedly, provirus in the buffy-coat cells of two cats that tested negative for the p27 antigen (a marker for viraemia) was also detected. In 597 Swiss cats, comparison of p27 antigen levels with PCR results revealed broad agreement. However, similar to the experimental situation, a significant number of animals (10%) was negative for the p27 antigen and FeLV-positive by PCR. These cats had a mean provirus load 300-fold lower than that of animals testing positive for the p27 antigen. In conclusion, an association between the provirus load and the outcome of FeLV infection was found. Detection of provirus carriers should contribute to further the control of FeLV. In addition, quantification of provirus loads will lead to a better understanding of FeLV pathogenesis and anti-retrovirus protective mechanisms.

Introduction

Feline leukaemia virus (FeLV) (Jarrett, 1975) is a retrovirus of veterinary importance (Hoover & Mullins, 1991; Hardy & McClelland, 1977) and is considered to be an important model for tumour research (Jarrett, 1975; Essex, 1982). Excellent vaccines for FeLV were developed several years ago (Jarrett & Ganiere, 1996; Sparkes, 1997) and these are commonly used in veterinary practices today. However, FeLV infection is still highly prevalent: 6% of 1526 sick and healthy animals sampled between 1996 and 1997 in Switzerland were positive for the FeLV p27 antigen (a marker for viraemia), as determined by ELISA (unpublished data).

Three main outcomes of infection are known (Hoover et al., 1975; Lutz et al., 1980; Hoover & Mullins, 1991; Rojko & Kociba, 1991): (i) persistently infected animals generally die within 3 to 4 years as a result of FeLV infection (McClelland et al., 1980), (ii) transiently FeLV-infected cats are able to overcome viraemia after a few weeks to months: the infection seems to be cleared and the animals are considered to be immune to re-infection, (iii) a third group of cats becomes immune to FeLV after the first contact with the virus. Although the susceptibility of cats to FeLV infection decreases with age (Hoover et al., 1975), the factors that determine which of the above-mentioned courses of infection will prevail is still unknown. We speculate that the initial load of virus and provirus may influence the outcome of retrovirus infections.

To study provirus load in both naturally and experimentally infected FeLV cats, we established a quantitative real-time fluorogenic probe-based FeLV PCR (TaqMan probe, Perkin
DNA polymerase, 15 pmol of each oligonucleotide primer, 1

This method makes use of the 5′ → 3′ exonuclease activity of Taq DNA polymerase (Holland et al., 1991; Lee et al., 1993; Lyamichev et al., 1993) and offers advantages such as absolute quantification, low DNA consumption, fast throughput of many samples and low risk of contamination (Heid et al., 1996). In addition, we report our nested FeLV PCR. We used these two PCR methods to detect and quantify FeLV provirus in naturally and experimentally infected cats. The results provide important new insights into the pathogenesis of FeLV infection.

**Methods**

**Samples and experimental design.** We collected EDTA blood samples from specified-pathogen-free (SPF) cats to assess the diagnostic specificity and sensitivity of the two newly established FeLV PCR methods. We used 30 FeLV-positive cats (Harlan) that had been intraperitoneally infected with 50 000 foci-forming units (ffu) of FeLV-A Glasgow and which had persistently tested positive for the p27 antigen, as judged by double-antibody sandwich ELISA (Lutz et al., 1983). A total of 45 FeLV-negative cats was also used (Ciba Geigy). All blood samples for ELISA were spun at 600 g for 10 min and plasma was stored at −20 °C. DNA for PCR, which was extracted from buffy coats (200 μl per sample) using the QIAamp Blood kit (Qiagen), was eluted into 100 μl AE buffer and was stored at −20 °C.

To observe the provirus load during infection, 15 15-week-old SPF cats (Harlan CPB) were infected intraperitoneally with 50 000 ffu of FeLV-A Glasgow. Blood samples were collected weekly for 15 weeks and the provirus load was determined by PCR. Cats were grouped into persistently antigenaemic cats and transiently or not antigenaemic cats, as judged by p27 antigen ELISA (Lutz et al., 1983). In addition, we determined anti-FeLV antibodies by both ELISA and Western blot using antigen (200 ng per test) that was purified on sucrose gradients, according to procedures described previously (Lutz et al., 1980, 1988).

Furthermore, we isolated DNA from 597 Swiss cats (as described above) to detect FeLV provirus in naturally infected cats and to determine the provirus loads in these cats. PCR results were compared with p27 antigen ELISA results.

**Detection of FeLV provirus by conventional nested PCR.** We combined two previously described PCR methods that amplify a conserved fragment in the FeLV U3 long terminal repeat (LTR) of exogenous FeLV (Rohn & Overbaugh, 1995; Jackson et al., 1993) to produce a nested FeLV PCR. The primers for the first round of PCR, 5′ AAAATTTAGCCAGCTACTGCAG 3′ (sense) and 5′ GAAGGTCGACCTGGGCCCCGGCT 3′ (antisense), amplify a 235 bp product. Primers were designed based on the FeLV-U3-1 and FeLV-U3-2B primer sequences (omitting the endonuclease restriction sites) described previously by Rohn & Overbaugh (1995). Reaction mixtures contained 1 × Thermophilic DNA polymerase buffer (Catalys), 2.5 mM MgCl₂, 0.2 mM of each of the four dNTPs (Catalys), 1 U Taq DNA polymerase (Sigma), 3.5 pmol of each oligonucleotide primer (Microsynth), 2 μg of total genomic DNA and ddH₂O in a total volume of 25 μl. Thermal cycling conditions were 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. The second round of PCR (nested) yielded a 166 bp product (Jackson et al., 1993). Reaction mixtures for the second round contained 1 × Thermophilic DNA polymerase buffer, 1.0 mM MgCl₂, 0.2 mM of each of the four dNTPs, 1.25 U Taq DNA polymerase, 15 pmol each of oligonucleotide primer, 1 μl of the first-round PCR product and ddH₂O in a total volume of 25 μl. Thermal cycling conditions were 3 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C. PCR assays were carried out in a DNA Thermal Cycler PTC-100 (MJ Research, distributed in Switzerland by Bioconcept). PCR products were analysed on ethidium bromide-stained agarose gels (1.8%).

**Detection and quantification of FeLV provirus by quantitative real-time PCR.** We designed primers and a probe that recognize the U3 LTR of exogenous FeLV-A (accession no. M12500; Stewart et al., 1986), but not endogenous FeLV sequences (Berry et al., 1988; Kumar et al., 1989), using the Primer Express software (Perkin Elmer). The target sequence was a conserved fragment of 74 bp within the region that is amplified by the nested PCR method. The primer sequences were 5′ ACCTGGGCCCCGGCT 3′ (15 bases; nt 2174–2188) for the sense primer and 5′ GCGGCCTTGAACCTCTCTGCT 3′ (20 bases; nt 2228–2247) for the antisense primer. The TaqMan FeLV probe, 5′ AGGCCAAGAACAGTTAACCCTCGGAT 3′ (Perkin Elmer) (26 bases; nt 2191–2216), was labelled at the 5′ end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). It was also phosphate-blocked at the 3′ end to prevent extension. Reaction mixtures consisted of 1.25 vol. 1 × TaqMan buffer A (Perkin Elmer) and 3.75 vol. 1 × PCR buffer II (Perkin Elmer), 5 mM MgCl₂, 0.2 mM of each of the four dNTPs (Catalys), 1.25 U Taq DNA polymerase (Sigma), 10 pmol each of oligonucleotide primer (Microsynth), 10 pmol TaqMan probe, 0.5 μg of total genomic DNA and ddH₂O in a total volume of 50 μl. Thermal cycling conditions were 3 min at 95 °C, five cycles of 0.5 min at 95 °C and 0.5 min at 64 °C followed by 40 cycles of 0.5 min at 85 °C and 0.5 min at 64 °C. We used an ABI Prism 7700 detection system (Perkin Elmer), which gives a threshold cycle (Cₜ) value for every sample. The Cₜ value is reached very rapidly if the amount of input target DNA is high. Quantification of the PCR is achieved by comparing the Cₜ value of the input DNA with the Cₜ value of a standard template DNA that is co-amplified in the same run.

**Analytical specificity of both PCR methods.** The analytic specificities of the nested and quantitative real-time PCR methods were confirmed by sequencing the PCR products following separation by gel electrophoresis and gel extraction with the Prep-A-Gene DNA Purification system (Bio-Rad). After ligation into the pT7Blue T vector (Novagen) using T4 ligase (Catalys), the resulting plasmids were used to transfect E. coli strain NovaBlue (Novagen). White colonies were screened for correct inserts by restriction enzyme analysis with HindIII and EcoRI (Catalys). The inserts of three clones were sequenced (Microsynth).

In addition, molecularly cloned viruses of the FeLV-A Glasgow (Stewart et al., 1986), FeLV-B Gardner–Arnstein (Elder & Mullins, 1983) and FeLV-C Sarma (Riedel et al., 1986) subgroups were used to determine the specificity of both PCR methods. Feline embryonic fibroblast (FEA) cells that harboured these FeLV subgroups were propagated in Dulbecco’s modified Eagle’s medium with 10% foetal calf serum. On day 15, 5 × 10⁵ cells were harvested and DNA was extracted using the QIAamp Blood kit (Qiagen).

**Analytical sensitivity of both PCR methods, production of the standard DNA template and linear range of the quantitative real-time PCR.** To assess the analytical sensitivity of the two PCR methods, FeLV-positive E. coli colonies (see above) were grown in LB medium containing 100 mg/l ampicillin (Fluka Chemie). Plasmid DNA was purified using the Qiagen Plasmid Midi kit (Qiagen) and linearized with HindIII. The concentration of the linearized DNA template was determined by measuring the optical density at 260 nm. The DNA template was then serially diluted tenfold in a solution of 100 ng/ml of...
calf thymus DNA (Sigma). The calculated copy numbers of DNA were confirmed by endpoint dilution experiments. Identical dilutions were used to compare the analytical sensitivity of the nested and quantitative PCR methods. Linear regressions ($y = a + bx$) and Pearson’s rank correlation coefficients ($r$) were calculated for the quantitative real-time PCR method.

**Efficiency of amplification and precision of quantitative real-time PCR.** To allow reliable quantification, the amplification efficiency of the FeLV standard DNA template and that of the same sequence within the viral genome should be approximately equal. We compared the efficiency of amplification by assessing the slopes ($s$) of the regression lines ($C_v$ versus dilution) obtained after PCR amplification of serial dilutions of either the standard or the genomic DNA templates of an experimentally FeLV-A-infected cat. The efficiency of amplification of each sample can be considered to be equal if the difference in the slopes ($A_s$) of the regression lines is less than $0.1$ (Gut et al., 1999).

The precision of the ‘within’ run of the quantitative PCR was assessed by multiple measurements ($n = 16$) of DNA from an experimentally FeLV-A-infected cat (#265) using a low (0.125 µg), an intermediate (0.5 µg) and a high (2 µg) quantity of DNA. The precision of the ‘in-between’ run was assessed by multiple assays ($n = 11$) with DNA from an experimentally FeLV-A-infected cat (#274) using 0.3 µg DNA per assay. Mean, standard deviations (SD) and coefficients of variation (CV) were calculated.

**Results**

**PCR methods to detect and quantify FeLV provirus**

For nested PCR, we combined two previously described primer pairs that amplify a highly conserved fragment of the U3 region of FeLV-A (Jackson et al., 1993; Rohn & Overbaugh, 1995). None of the additional bands (approximately 100 and 210 bp) that were described originally by Jackson et al. (1993) were observed (Fig. 1a). The quantitative real-time PCR was designed to amplify a 74 bp fragment within the same U3 region. The diagnostic specificity of both methods was 100%: 45/45 samples from non-infected SPF cats tested negative for FeLV by both nested and quantitative PCR. Endogenous FeLV-related DNA sequences were not amplified by either method. The diagnostic sensitivity for FeLV-A detection was 100%: 30/30 samples collected 10 weeks after experimental FeLV-A infection from cats positive for the p27 antigen tested positive by both nested and quantitative PCR.

Both PCR methods were evaluated for analytic specificity by sequencing the PCR products obtained from FeLV-infected cats. The sequences were identical to that of FeLV-A (data not shown). We used molecular clones to determine the ability of both PCR methods to amplify FeLV from different subgroups. The nested PCR method recognized all three FeLV subgroups, while the quantitative real-time PCR amplified FeLV-A Glasgow (2.9 × 10^{-2} provirus copies per FEA cell) and FeLV-B Gardner–Arnstein (0.55 provirus copies per FEA cell), but not FeLV-C Sarma.

The analytical sensitivity of the two PCR methods was compared by amplifying a tenfold serial dilution of the cloned standard DNA template. Both methods detected between 0.36 and 3.6 copies of the template DNA (Fig. 1). Sensitivity was highly reproducible: figures are representative of the results of several experiments yielding corresponding results ($n = 4$ for nested PCR; $n = 20$ for quantitative real-time PCR).

The quantitative real-time PCR method was also tested for amplification efficiency. The efficiency of amplification of standard and genomic DNA should be approximately equal to allow valid quantification. Efficiencies can be considered to be equal if the difference in the slopes ($A_s$) of the regression lines ($C_v$ versus dilution of input DNA) obtained with serial dilutions of different DNA is less than $0.1$ (Gut et al., 1999). We compared the regression lines of the cloned standard DNA template ($s = -3.31, r = 0.990$) with that of genomic DNA from an experimentally FeLV-A-infected cat ($s = -3.39, r = 0.986$). The $A_s$ value was $0.08$. Therefore, the method is considered to be equally efficient for both standard and sample DNA and quantification using the DNA standard is expected to be valid.

Quantitative PCR was further evaluated for linear range and precision. By amplifying a serial dilution of the standard DNA template, a wide linearity of the method was demonstrated (10 orders of magnitude, Fig. 1b). Precision was...
assessed by multiple measurements of DNA samples of experimentally FeLV-A-infected cats and was found to be high: CV of the $C_T$ values ranged from 0–9 to 1–0% for the ‘within’ run and from 0–9 to 4–4% for the ‘in-between’ runs.

**Provirus loads after experimental FeLV infection and correlation with the outcome of infection**

Cats experimentally infected with FeLV-A were observed for 15 weeks after infection. Animals were grouped according to their p27 antigen ELISA results (Fig. 2). If the value of absorbance at 260 nm reached 5% or more of the positive control, ELISA results were defined as positive. Five animals showed a regressive value and viraemia, two animals never had a positive ELISA result and three animals were transiently positive for p27. Ten animals became persistently infected and showed progressive viraemia. Animals started to become positive by PCR from 1 to 2 weeks after experimental infection (Fig. 2). Interestingly, cats that remained negative for the p27 antigen became positive by PCR.

Copy numbers of provirus DNA per cell (Fig. 3) were calculated by dividing the number of copies of U3, as determined by real-time PCR, by two. This figure was then divided by the number of cells, as deduced from the amount of DNA used per reaction. Cats with a regressive course of infection had mainly low copy numbers of provirus DNA (Fig. 3c; weeks 1–15, 0–22 ± 1–7 copies per cell). Although cats that tested negative for the p27 antigen became positive by PCR, their virus loads never exceeded 1 copy per 100 leukocytes (weeks 1–15, 0–08 ± 0–07 copies per cell). One transiently infected cat that tested positive for the p27 antigen had a major peak of provirus in week 4 (15 copies per cell). The other two transiently positive cats showed only a minor peak of provirus around week 4 (0–2 and 0–4 copies per cell, respectively). Thereafter, all three transiently infected cats had low copy numbers until the end of the experiment (< 0–1 copy per leukocyte).

In contrast, persistently infected cats had a higher mean provirus load (weeks 1–15, 1–4 ± 2–7 copies per cell). The provirus load of these animals showed the first peak between weeks 3 and 5 (two animals had over 10 copies per cell in week 4) and a decrease thereafter (Fig. 3d). A second peak was found between weeks 10 and 14 (the same two animals had over 10 copies per cell in week 12). The magnitude of the second peak was directly correlated with that of the first peak (Pearson’s rank test $r = 0–9191, P = 0–0002$; data not shown). Significant differences in provirus load between persistently infected cats and animals with a contained course of infection were found in weeks 3, 6 and 7, and from week 10 onwards (Mann–Whitney U-test $P < 0–05$; Fig. 3c, d).

ELISA was used to determine antibody levels during the weeks indicated in Fig. 3(e, f). In addition, the specificity of antibodies in weeks 0, 4, 8, 12 and 15 after experimental infection was determined by Western blot (data not shown). To some extent, all animals developed antibodies specific for FeLV proteins, indicating that infection was successful in all animals. Cats with regressive viraemia developed significantly higher antibody levels than persistently infected cats (Mann–Whitney U-test $P < 0–05$; Fig. 3e, f). Antibody levels early in infection were indirectly correlated with provirus loads. Cats with high levels of antibody in week 3 had low provirus loads.
**FeLV: provirus load and outcome of infection**

**Regressive, contained viraemia versus progressive, persistent viraemia after experimental FeLV infection.**

(a) FeLV p27 antigen load expressed as a percentage of the positive control (pc), as determined by ELISA.

(b) FeLV provirus load, as determined by real-time PCR.

(c) Antibodies against FeLV expressed as a percentage of the pc, as determined by ELISA.

Regressive, contained viraemia: cats testing transiently positive or negative for the p27 antigen (n = 5, cats 122, 133, 153, 161 and 181; see also Fig. 2). Progressive, persistent viraemia: cats persistently testing positive for the p27 antigen (n = 10; remaining cats in Fig. 2). Data are shown as box plots. Boxes extend from the 25th to the 75th percentile; a horizontal line represents the median and the error bars extend down to the smallest and up to the largest value. *P* values indicate the significant difference between the regressive and the progressive group (Mann–Whitney U-test *P* < 0.05).

PCR results in naturally infected cats and comparison with p27 antigen ELISA results

We collected blood samples from 597 Swiss cats and analysed them for the presence of provirus and p27 antigen. PCR results corresponded well with ELISA results: all of the positive ELISA samples were positive by PCR (n = 41) and all of the negative PCR samples were negative by ELISA (n = 495). Interestingly, 10% of the cats that were positive by PCR were negative for the p27 antigen (n = 61). These samples were re-evaluated several times and negative controls were carried in all the experiments to assure that these results were not due to contamination. The majority of these cats (71%) did not show any clinical signs of infection. Provirus load was determined in cats that were positive for the p27 antigen and in animals that were negative for the p27 antigen but positive by PCR. The latter group had significantly lower (by a factor of 300) provirus loads (0.03 ± 0.14 provirus copies per cell) than cats that were positive by ELISA (10 ± 47 provirus copies...
per cell, Mann–Whitney U-test \( P < 0.0001 \). The two PCR methods revealed satisfactory agreement: 86 samples were shown to be positive for FeLV and 492 were shown to be negative by both methods. Some discrepant results were found: one cat was found to be positive by quantitative PCR and negative by nested PCR and 15 animals (2.5%) were positive by nested PCR but negative by quantitative PCR. Detailed analysis of the naturally infected cats will be presented elsewhere.

**Discussion**

To study the presence and load of provirus in experimentally and naturally FeLV-infected cats, we established a nested PCR and a quantitative real-time PCR. Both methods recognize fragments within the U3 region of FeLV-A and proved to be sensitive, specific and highly reproducible. These PCR methods amplify exogenous FeLV, but not endogenous FeLV-related DNA sequences. In addition, real-time PCR allows quantitative results and fast throughput of large numbers of samples.

We discovered healthy cats that were positive for FeLV provirus and negative for the p27 antigen, which have not been described previously: (i) 10% of 597 Swiss cats examined were negative for the p27 antigen, but were FeLV-positive by PCR, (ii) two experimentally FeLV-A-infected cats testing negative for the p27 antigen became positive for provirus at 1 week after virus exposure, (iii) experimentally FeLV-A-infected cats that were transiently infected remained positive for provirus, even after clearing the p27 antigen from their blood. This contradicts the observations by Miyazawa & Jarrett (1997) and Jackson et al. (1996) who reported a good agreement between p27 antigen ELISA and PCR results and no cats that were negative for the p27 antigen and positive by PCR. As repeated experiments yielded identical results and proper controls were always included, we are confident that the discrepancies between ELISA and PCR are not due to contamination. Moreover, we hypothesize that the cats detected in the field that were positive by PCR and negative for the p27 antigen had, in fact, been infected with FeLV but were able to overcome antigenaemia. Alternatively, some of these cats might have been in the early phase of infection. Interestingly, these cats had significantly lower provirus loads than their p27 antigen-positive counterparts, which parallels our experimental studies in which cats that overcame antigenaemia had lower provirus loads than persistently infected cats. We did not analyse the 597 serum samples for the presence of antibodies to FeLV, nor did we determine whether virus production could be reactivated. The biological importance of this rather large population (10% of Swiss cats sampled) cannot yet be addressed, but the quantitative PCR method will allow both the observation of these cats and the determination of virus clearance. Provirus persistence could also be beneficial: for the non-retrovirus lymphocytic choriomeningitis virus of the mouse, it was postulated that persistence of the stably integrated DNA might contribute to the maintenance of the immune system memory (Klenerman et al., 1997).

The provirus loads in our experimentally infected cats (average of all cats from weeks 1–15, 1.1 ± 3.3 copies per cell) were similar to those found by semi-quantitative PCR in cats infected with the two major FeLV-FAIDS genotypes, 61C and 61E (Quackenbush et al., 1996a, b). Only two persistently infected cats twice showed provirus loads exceeding 10 copies per cell (weeks 4 and 12 post-infection). An explanation for this might be found in the high numbers of unintegrated viral DNA (UVD), which is not distinguishable from integrated provirus by our methods. UVD appears to be obligatory to the lifecycle of retroviruses and is detected in *vitro* a few days after infection of cultured cells with high virus multiplicity (Mullins et al., 1986). This phenomenon is not usually observed in *vivo*, since few cells in a sample are infected synchronously immediately before collection.

Provirus loads in the antigen-positive, naturally FeLV-infected cats were higher than those in the experimentally infected cats. This may be explained by the fact that experimental infection was performed by intraperitoneal injection of infectious FeLV, which does not represent the typical mode of transmission (via the oropharynx) under natural conditions. Another explanation may again be UVD. Persistent UVD was demonstrated in high concentrations (15–50 copies per cell) mainly in the bone marrow, but also in intestine and lymphoid tissues of FeLV-FAIDS-infected cats in the terminal stages of disease (Hoover et al., 1987; Mullins et al., 1986). So far, UVD has not, or only to a small extent, been found in cats with diseases induced by other FeLV strains and in healthy FeLV-FAIDS-infected cats (Mullins et al., 1986, 1991). Another additional explanation for the higher provirus loads in naturally infected cats could be the unknown, but supposedly longer, duration of infection in naturally infected cats than in experimentally infected animals. In the latter animals, the load of provirus was determined early in infection (up to week 15). Increased duration of infection might lead to an increase of the provirus load in cats that persistently test positive for the p27 antigen. Provirus burden gradually increased throughout the course of FeLV-FAIDS infection and the fraction of cells harbouring FeLV antigen was higher in chronically infected cats than in acutely infected animals (Quackenbush et al., 1996a, b).

The primers and the probe for quantitative PCR were designed to be specific for FeLV-A sequences. Both the quantitative and the nested PCR methods detected an FeLV-B molecular clone, but only the nested PCR method recognized FeLV-C Sarma. As all cats infected with FeLV are known to harbour FeLV-A (Jarrett et al., 1978), false-negative results were not expected. However, when naturally infected Swiss cats were analysed by both PCR methods, discrepant results (positive by nested PCR but negative by quantitative real-time...
PCR) were found in 15 cats. We suspect that these cats harboured FeLV-C. Virus isolation, interference and neutralization assays (Sarma & Log, 1973) as well as sequencing of the variable regions in env (Riedel et al., 1986, 1988) will be necessary to address this question. To avoid false-negative results when assessing naturally infected cats, we recommend the use of nested PCR or to enhance real-time PCR by a second probe.

Provirus was quantified in buffy-coat cells of cats during the early stage of experimental FeLV-A infection. We speculated that the initial provirus load might influence the outcome of infection. Challenged animals were grouped into persistently infected cats and cats with a regressive, contained viraemia (transiently antigenaemic or p27 antigen-negative). The persistently, progressively infected animals had an initial peak of high provirus loads at around week 4 after challenge infection. A similar, but less pronounced initial peak, was also found in transiently antigenaemic cats, but not in cats that tested negative for the p27 antigen. Furthermore, animals with an initially high provirus load had high antigenaemia and provirus load later in the experiment. Therefore, we hypothesize that the initial provirus load may be associated with the outcome of infection. However, to prove this hypothesis, a higher number of cats would have to be studied over a longer period of time.

To determine what factors might influence the magnitude of the initial provirus load, we determined FeLV-specific antibody production. Differences in humoral immune responses between cats with progressive and regressive antigenaemia were initially evident 3 weeks after virus exposure and coincided with the appearance of differences in provirus loads. Cats with contained antigenaemia displayed a more pronounced humoral immune response and lower provirus loads than animals with progressive antigenaemia. Consequently, a cat that is capable of immunologically controlling the initial peak of provirus may be able to effectively overcome viraemia. By lowering the initial provirus load to under a certain threshold or by augmenting the early humoral immune response, e.g. by vaccination, cats may be protected from persistent infection. Furthermore, antibodies seem to contribute to the control of infection after the initial peak of provirus: cats with high antibody levels had low or undetectable antigen and provirus throughout the remainder of the experiment and vice versa. It must be assumed that other factors also play an important role in defeating FeLV, such as the cellular immune response (Flynn et al., 2000) or a more or less pronounced natural resistance of the host. One of the transiently antigenaemic cats showed initial low levels of anti-FeLV antibodies, was antigen-positive and had a high provirus load (as high as those of the persistently infected cats). Nevertheless, this cat developed high antibody levels by week 4, became antigen-negative and the provirus load decreased; the animal kept this status until the end of the experiment. It is unclear what the ‘rescue mechanism’ was in this cat that showed all the signs of an upcoming persistent infection.

In conclusion, detection of provirus-positive carriers and the determination of the provirus loads provide important additional parameters that allow the more precise characterization of the pathogenesis of FeLV infection in naturally and experimentally infected cats. In addition, these parameters may be valuable to study the mechanisms that lead to protection after FeLV vaccination.

This study was supported by a grant from the United Bank of Switzerland on behalf of a customer, by the state of Zurich and by the European concerted action on feline AIDS. We thank Dr O. Jarrett who kindly provided the molecular FeLV clones. We further thank Dr R. Wicki (Perkin Elmer, Rotkreuz, Switzerland) for the support with the primer sequences. The representative of IAMS Company, Sieber-Hegner & Co, Zurich, Switzerland kindly donated cat food. We thank Dr K. Bauer, A. Chalmers, P. Fidler, T. Konersmann, Dr C. Leutenegger, C. Mislin, D. Wegmann and C. Wolfensberger for excellent assistance. R.H.-L. is supported by a grant from the Swiss National Science Foundation (Fellowship number 823A-50315) and is the recipient of a Scholar Award from the Friends of Switzerland, Inc.

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Received 26 September 2000; Accepted 8 March 2001