Detection of Feline Immunodeficiency Virus (FIV) Nucleic Acids in FIV-Seronegative Cats

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A study was undertaken to determine the rate of viral transmission among naive specific-pathogen-free (SPF) cats living in close contact with feline immunodeficiency virus (FIV)-infected cats. Twenty SPF cats were housed in the same rooms with experimentally FIV-infected seropositive and virus culture-positive cats for 2 to 4 years and were monitored for the presence of FIV nucleic acids and antibodies. Only 1 of the 20 cats became seropositive and virus culture positive and developed signs of disease. Genomic DNA from bone marrow and peripheral blood mononuclear cells (PBMC) of 10 of 19 healthy-appearing seronegative cats became positive for FIV DNA by the polymerase chain reaction. Twenty-eight SPF cats housed as groups in separate quarters and never exposed to FIV-infected cats were uniformly negative for FIV DNA. FIV RNA transcripts were detected in concanavalin A-stimulated PBMC cultures from 4 of 10 FIV DNA-positive, seronegative cats by in situ hybridization. PBMC from three of four naive SPF cats acquired FIV nucleic acids after the cats were transfused with blood and bone marrow from FIV genome-positive, seronegative donors. Three of five FIV-seronegative cats housed for years with naturally FIV-infected cats in a private household were also found to harbor FIV DNA, indicating that the same phenomenon occurred in the field. These findings demonstrate that cats living in close contact with FIV-infected seropositive cats can acquire FIV nucleic acids without developing detectable levels of serum antibodies or disease.

Infection with the human immunodeficiency virus (HIV) usually results in a transient mononucleosilalic disease several weeks after exposure and development of detectable levels of serum antibodies to the virus (18). HIV can be readily isolated from the blood of most seropositive people, and seropositivity and viremia are generally assumed to be synonymous. A more unconventional pattern of infection appears to be emerging, according to recent studies of HIV infection, especially among sexual contacts and children born to seropositive mothers. A portion of outwardly healthy seronegative sexual contacts of patients with AIDS test positive for HIV by polymerase chain reaction (PCR) and/or in situ hybridization (11, 20, 29). Similar findings for some of the seronegative children born to HIV-infected mothers have been reported elsewhere (36). Circulating B lymphocytes from some seronegative people can produce HIV-specific antibody following in vitro stimulation by pokeweed mitogen, corroborating genome testing (13).

Two possible explanations for the phenomenon of HIV genome-positive, seronegative individuals are available. The first is that seroconversion is merely delayed longer than usual in some HIV-infected individuals (8, 10, 11, 39). The second possibility is that HIV infection may interact with the host and vice versa in an unconventional manner. Many factors, such as the route of infection, amounts of the infecting virus, viral variants, or host immune response to the infecting virus, may influence the time period needed for seroconversion and disease development. The occurrence of seronegativity in latent infection is still a debatable issue.

Availability of animal models may be crucial to study the significance and mechanisms of HIV genome positivity accompanied by seronegativity.

Feline immunodeficiency virus (FIV) is a lentivirus that causes an immunodeficiency syndrome in cats (25). The pathogeneses of FIV and HIV infections in their respective hosts are remarkably similar (2, 6, 19, 23-27, 33, 42). FIV can be readily isolated from seropositive, symptomatic cats. However, FIV isolation from cultured peripheral blood mononuclear cells (PBMC) of stray cats with no detectable antibody has been reported elsewhere (9). Cats experimentally infected with FIV develop a transient flulike illness associated with seroconversion before entering a long latent period of outward normalcy. It has been assumed that all infected cats will eventually become seropositive (22, 41, 42). In one study, all experimentally infected cats except one seroconverted within several weeks; the cat that did not seroconvert in that period became seropositive only after a period of 14 months (42).

The major route of transmission of FIV appears to be biting (42). Free-roaming male cats engaged in territorial behavior are at the highest risk for infection in nature. Seronegative cats housed together with infected cats usually remain seronegative, probably because aggressive behavior is largely suppressed when cats are confined together in a stable household. Until recently, it had been assumed that all FIV-seronegative cats living with seropositive animals are uninfected. We report here on the frequent presence of the FIV genome in persistently seronegative cats that have been housed for prolonged periods with seropositive FIV-infected cats.
which had detectable contained infected and uninfected the contact-exposure during blot

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Cats housed together (cat no.)</th>
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</tr>
<tr>
<td>3</td>
<td>7* 9* 32* 33 34 35 2433* 2839*</td>
<td>115</td>
<td>42 54</td>
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<tr>
<td>13</td>
<td>462 466 468 516 518 520</td>
<td>M2 2</td>
<td>12</td>
</tr>
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</table>

* Groups 1 and 9 to 13 contained only uninfected SPF cats. Groups 2 to 8 contained infected and uninfected cats together.

<table>
<thead>
<tr>
<th>Cat no.</th>
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<tbody>
<tr>
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</table>

**MATERIALS AND METHODS**

**Experimental animals.** Specific-pathogen-free (SPF) cats never exposed to FIV-infected animals were obtained from the breeding colony of the Feline Retrovirus Research Laboratory, School of Veterinary Medicine, University of California, Davis. The cats were seronegative for FIV antibody by an indirect immunofluorescence assay (IFA), an enzyme-linked immunosorbent assay (ELISA), and Western blot (immunoblot) analysis.

**Experimental design.** A total of 69 SPF cats were used in the present study at the University of California, Davis. Of these, 21 cats were experimentally infected with FIV and maintained for periods ranging from 32 to 54 months. A total of 20 age- and sex-matched littermates that were not given the virus were housed together in open rooms with the infected animals and served as contact-exposure controls (groups 2 to 8 [Table 1]). A total of 28 additional age-matched cats from the same bloodline were housed in identical quarters but out of contact with any FIV-infected animals. The 69 cats were dispersed among 13 groups, as shown in Table 1. Most rooms contained four to eight cats, of which approximately half were FIV seropositive. Six groups (group 1 and groups 9 to 13) consisted solely of uninfected, SPF cats that had never contacted FIV-infected cats and served as negative controls. The cats were allowed to roam freely in each room and shared litter pans and food and water dishes.

In a separate study, we monitored a group of 17 cats housed together over a period of 5 to 6 years in a private home in Petaluma, Calif., from which FIV was first isolated (25). Most of these cats were homeless or feral prior to obtaining shelter in this household. Twelve of the cats in this household had naturally acquired FIV infection at the beginning of the study. Like the experimental cats, seropositive cats were free to roam and shared pans for litter, food, and water with seronegative cats.

**FIV infection.** For the experimental FIV infection, SPF cats were inoculated intraperitoneally or intravenously with infectious blood, plasma, or PBMC culture fluid (42). The mode of infection and the type of viral inoculum for individual cats in this group have been previously described (33).

All of those cats became positive for FIV antibodies within 2 to 4 weeks.

**SeroLOGY.** Sera were probed for antibodies to FIV by Western blots and by an IFA that used FIV-infected Crandell feline kidney (CfFK) cells as the substrate (41). FIV-infected Crandell cells were trypsinized and suspended in growth medium containing Leibovitz’s L-15 medium and Dulbecco’s modified Eagle medium (1:1), and the cell suspension was put onto Teflon-templated multislot slides. The slides were incubated for 5 h at 37°C in a humidified chamber to allow cells to adhere and were then fixed with acetone. Serum samples from cats were incubated on these fixed slides at a dilution of 1:10 in phosphate-buffered saline (PBS) solution for 1 h at 37°C. After being washed, the slides were incubated with fluorescein-conjugated rabbit anti-cat immunoglobulin G antibodies (Antibodies Inc., Davis, Calif.) for 1 h. The slides were washed two times with PBS and then counterstained with 0.01% Evans blue, and coverslips were mounted with 50% glycerol in PBS and read by using a fluorescence microscope. The serum samples were considered positive for FIV antibodies when giant cells and infected cells had typical membrane and cytoplasmic fluorescence. Serum samples from experimentally FIV-infected cats were used as positive controls. Sera from uninfected or FIV-infected SPF cats were used as negative controls in each assay.

To further confirm the serological results of IFA, the sera of cats in the present study were analyzed by Western blot. FIV was propagated in cat PBMC cultures or in FL 4 feline T cells (40). Virus was pelleted from tissue culture medium and purified through a 10 to 50% continuous sucrose gradient. Viral proteins were separated by electrophoresis in 12.5% polyacrylamide gels and transferred to nitrocellulose paper. Strips of the paper were used as substrates for immunoblots. Serum samples were diluted 1:100 and incubated with Western blot strips in separate glass tubes for 12 h at 37°C. The blots were washed individually and incubated with peroxide-conjugated rabbit anti-cat immunoglobulin G (Cappel Laboratories, Cochranville, Pa.). The strips were washed and incubated with 0.05% dianisobenzidine and 0.01% H2O2 in 0.1 M Tris (pH 7.4) to visualize the antigen-antibody binding. The reaction was stopped by addition of excess water when visible bands appeared. The molecular weights of the bands were determined from the amido-black-stained standard molecular weight markers on the same Western blot. Each Western blot contained positive and negative control sera. Serum samples from all of the cats in the present study were analyzed by the immunoblots for the FIV antibodies. Sera from 15 uninfected SPF cats and 10 experimentally FIV-infected cats were used as negative and positive controls, respectively.

**Enumeration of lymphocyte subsets.** Fluorescein-conjugated murine monoclonal antibodies to feline CD4+ (1) and feline CD8+ (16) T-cell surface markers were used for enumeration of T-lymphocyte subsets by flow cytometric analysis as described elsewhere (4, 26, 33).

**Virus isolation.** Blood samples were defibrinated with glass beads, and the PBMC were harvested on Ficoll gradients (4) and cultured with 5 μg of concanavalin A (ConA) per ml and 100 U of human recombinant interleukin-2 (courtesy of Cetus Corporation, Emeryville, Calif.) per ml. On day 3, the stimulated cells were cocultivated with PBMC from a non-infected donor cat. PBMC from uninfected SPF cats were used as negative controls. Cultures were maintained, and culture supernatants were monitored weekly for reverse transcriptase (RT) activity (42) and for viral antigens by...
ELISA on the basis of antigen capture. PBMC from experimentally FIV-infected cats usually became virus positive for the RT activity and by ELISA within 2 to 3 weeks in culture. PBMC cultures from FIV-seropositive cats were monitored for 6 to 8 weeks, while PBMC from FIV-negative animals were maintained in culture for 12 to 14 weeks. The rationale for longer-term culture of PBMC from FIV-seronegative cats was to give slowly growing or defective FIV variants time to propagate to detectable levels.

An ELISA to detect FIV p24 antigen in culture supernatant was developed. Microtiter plates (96-well Immulon 2; Dynatech, Chantilly, Va.) were first coated with 1 μg of affinity-purified goat anti-cat immunoglobulin G (Kirkegaard & Perry, Gaithersburg, Md.) per ml in ELISA coating buffer (0.1 M Na2CO3 [pH 9.6]). The plates were washed ELISA wash solution (0.15 M NaCl, 0.05% Tween 20) and incubated with a blocking buffer consisting of PBS with 0.2% Tween 20 and 5% powdered dry milk for 1 h at room temperature. The plates were then washed and reacted with anti-FIV cat sera (with high titers of FIV antibodies) diluted 1:1,000 in ELISA dilution buffer (0.05 M Tris, 0.15 M NaCl, 0.001 M EDTA, 0.05% Tween 20, 0.1% bovine serum albumin [pH 7.4]). The plates were then washed with ELISA wash buffer. 7.400-μl volumes of PBMC culture supernatants was incubated with 100 μl of ELISA dilution buffer containing 0.2% Tween 20 for 1 h at 37°C. The plates were washed and incubated with an appropriate predetermined dilution of biotinylated anti-FIV p24 mouse monoclonal immunoglobulins, 43-I9B and 43-I2E, in ELISA dilution buffer for 1 h at 37°C. The plates were washed and incubated with streptavidin peroxidase (Kirkegaard & Perry) for 20 min at 37°C. After the plates were washed, tetramethylbenzidine (15 μg/ml in 0.05 M citric acid [pH 4.0] with 0.15% H2O2) was used as a substrate to develop color. The color development reaction was stopped after 5 to 15 min with 50 μl of 1 N sulfuric acid, and the optical density at 450 nm of wells was read on an ELISA reader (Dynatech). Unless otherwise stated, all volumes were 200 μl. Culture supernatants from chronically FIV-infected CrFK cells, supernatants from PBMC cultures, and polyacrylamide gel electrophoresis-purified FIV p24 antigen were used as positive controls. Culture supernatants from uninfected CrFK cells and PBMC were used as negative controls. The assays had a sensitivity of 50 ng of FIV p24 per ml.

**Detection of FIV DNA by PCR.** Proviral DNA of FIV was amplified by using two sets of primer pairs specific for the gag (bp 929 to 948 and bp 1385 to 1394) and env (bp 6386 to 6405 and bp 6666 to 6685) regions of the FIV genome (26, 34). Primer pairs were selected from FIV DNA sequence data (31). Specific 32P-labeled oligonucleotide probes (bp 1277 to 1308 for gag and bp 6564 to 6589 for env) were prepared and used to identify the amplified products by Southern blotting.

Proviral DNA was extracted from PBMC and bone marrow. Samples of approximately 200 to 300 ng of purified DNA were subjected to 30 cycles of PCR amplification in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) with the thermostable Taq DNA polymerase (Promega Biotechnology). The DNA samples were denatured at 94°C for 1 min, renatured at 55°C for 1 min, and elongated at 72°C for 1.5 min. Amplified DNA fragments were separated by electrophoresis in a 1.5% Nusieve GTG agarose-1.5% agarose gel placed into nitrocellulose. Southern blots were hybridized to 32P-labeled oligomer probes at 55°C for 16 h.

The genomic DNA samples from bone marrow and PBMC of all the cats in the present study were analyzed for the presence of FIV DNA following PCR. Blood samples for serology and for PCR analysis were collected at the same time from seronegative, PCR-positive cats as well as from control cats. Genomic DNA samples from experimentally FIV-infected cats and uninfected SPF cats were used as positive and negative controls, respectively. To determine the level of false positives in the PCR assay, samples from 28 SPF cats were used as negative controls in the PCR analysis by using the same set of primers and experimental conditions. At least three positive and three negative controls were included in every PCR assay. Blood samples drawn at three different times from seronegative PCR-positive cats were analyzed for FIV DNA by PCR analysis. The samples were collected over a period of 1 year.

**Detection of FIV RNA by in situ hybridization.** PBMC cultures were probed for FIV RNA by an in situ hybridization protocol modified from previously published procedures (5). Cultures were established from 2 ml of whole blood following Ficoll gradient centrifugation and maintained for 6 days in RPMI 1640 medium containing interleukin-2 and ConA. The cells were then pelleted by low-speed centrifugation, washed with PBS, and spotted onto glass microscope slides. All of the slides were fixed in 4% paraformaldehyde, washed in PBS, treated with proteinase K (1 to 5 μg/ml), dehydrated in alcohol, and hybridized with a 35S-labeled FIV DNA probe (specific activity, ≥1010 cpm/μg) in a solution containing 50% deionized formamide, 10% dextran sulfate, 50 mM NaH2PO4, 0.6 M NaCl, 0.5 mM EDTA, 1× Denhardt’s solution, 75 μg of Escherichia coli tRNA per ml, 100 μg of salmon sperm DNA per ml, and 20 mM dithiothreitol in a humidification chamber at 40°C.

A 9.2-kb FIV genomic DNA fragment containing entire gag, pol, and env regions was radioactively labeled by nick translation to generate FIV DNA probe (3 × 107 cpm/ml), which was added to the hybridization solution. To remove unhybridized probe, the slides were washed four times with wash buffer containing 50% formamide and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 40°C, followed by four washes in 2× SSC at 37°C. Autoradiography was performed with NTB2 emulsion (Eastman Kodak, Rochester, N.Y.) for 13 to 17 days. Slides were developed with Kodak D-19 developer and counterstained with hematoxylin to visualize the cells. Controls included FIV-infected and uninfected CrFK cells and PBMC from uninfected control cats and from FIV-infected seropositive cats.

**Whole-blood lymphocyte stimulation microassay.** The assay performed was a modification of established procedures (4, 33). Blood samples were collected by jugular venipuncture into sterile tubes containing preservative-free sodium heparin and incubated either with ConA (6 μg/ml) or pokeweed mitogen (3 μg/ml) or without mitogen (with unstimulated controls reflecting spontaneous proliferation). [3H]thymidine incorporation into DNA was measured. Each of the three treatments (ConA, pokeweed mitogen, and no stimulation) was replicated three times for each blood sample. Mean values were calculated for use in statistical analysis.

**Depletion of CD8+ cells from PBMC.** PBMC, with or without culturing in the RPMI media with ConA and interleukin-2, were incubated with mouse monoclonal antibodies specific for the feline CD8+ T-cell marker (Southern Biotechnologies Associates Inc., Birmingham, Ala.). The cells were washed and incubated with goat anti-mouse immunoglobulin associated with magnetic beads (Dynal Inc., Great Neck, N.Y.), and the CD8+ T cells were removed by a magnet. The CD8+ cell-depleted PBMC were cultured in
FIG. 1. Detection of FIV antibodies in serum samples by Western blot analysis. Western blots containing viral antigens were reacted with sera from (i) FIV-infected cats (cat no. 20, 21, 22, 26, 32, and 2428) (lanes 1 to 6), (ii) uninfected SPF cats (cat no. 1, 2, 3, and 3924) (lanes 7 to 10), (iii) contact control cats (cat no. 23, 3923, 2840, 25, 37, 19, 8, 38, 16, and 12) (lanes 11 to 20), (iv) cats prior to transfer study (cat no. 90458, 90468, 90490, and 90508) (lanes 21 to 24), and (v) cats posttransfer study (cat no. 90458, 90468, 90490, and 90508) (lanes 25 to 28).

RPMI medium, and supernatants were assayed for the presence of viral p27 core antigen and RT activity.

Transfer of blood and bone marrow. Blood (4 ml) and bone marrow aspirates (0.5 ml) were obtained from four FIV genome-positive, seronegative cats (no. 16, 19, 23, and 37) and injected into the femoral marrow cavities of four respective recipient cats (no. 90458, 90468, 90490, and 90508). PBMC from the recipient SPF cats were negative for the presence of FIV nucleic acids prior to the transfer by both PCR and in situ hybridization assays. Blood samples were obtained at weeks 2, 4, 6, 8, 10, and 19 posttransfer. Sera were tested for FIV antibodies by ELISA and Western blots (33, 41). The genomic DNAs from PBMC samples were examined for FIV DNA following gene amplification by PCR. PBMC were cultured for 6 to 10 days after ConA stimulation and examined for the presence of FIV nucleic acids by in situ hybridization.

Statistical analysis. Differences in mean values of lymphocyte numbers were evaluated for statistical significance (P < 0.05) by Student's two-tailed t test and by a corresponding nonparametric method, the Mann-Whitney U test (3).

RESULTS

Clinical, serological, and virological status of cats. The unexposed control cats in groups 1 and 9 to 13 (Table 1) remained healthy and seronegative for FIV antibody by IFA and Western blot analysis (Fig. 1, lanes 7 to 9). They maintained normal CD4+ -to-CD8+ T-cell ratios (see Table 4), and their ConA-stimulated PBMC were negative for RT activity.

The 21 cats in groups 2 to 8 that were experimentally housed for several years with seropositive, FIV-infected cats in the laboratory environment were housed in the University of California, Davis, facility. Blood samples for PCR analysis and serology were obtained at the same time.

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The 21 cats in groups 2 to 8 that were experimentally
infected with FIV remained grossly asymptomatic through the 32- to 54-week course of the study. The cats seroconverted for FIV antibodies within 2 to 4 weeks following FIV infection and remained consistently FIV seropositive for the remainder of the study. Sera from these cats recognized the major gag proteins of 24 and 17 kDa each on Western blot (Fig. 1, lanes 1 to 6). Antibodies to a gag precursor protein of 49 kDa, an RT protein of 62 kDa, and an envelope
VOL. 66, FIV Seronegative, vitrō protein glycoprotein core of cultures from capture ELISA. a as negative 2). analysis (Fig. 1, with of positive remained to prior (no. 6, 7, 9, 10, 17, 2433, 2436, 2836, ConA 25,573 0.05) a PWM, C

The Detection of FIW CD4+ pokeweed mitogen; T, mean differs significantly (P < 0.05) and by day 6, there were 10 to 20 infected cells per 2,000 cells, and by day 13 there were 80 to 100 FIV-infected cells per 2,000 cells.

Detection of lymphocyte subsets. There were no significant differences in CD4+ or CD8+ T-cell parameters between seronegative uninfected and FIV genome-positive, seronegative cats (P > 0.05) (Table 4). The seropositive cats as a group exhibited a significant decrease in the percentages

TABLE 4. Enumeration of lymphocyte subsets

<table>
<thead>
<tr>
<th>Cat group</th>
<th>CD4+ lymphocytes</th>
<th>CD8+ lymphocytes</th>
<th>CD4+-to-CD8+ T-cell ratio</th>
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<td></td>
<td>No. of cells/μl</td>
<td>% Total lymphocytes</td>
<td>No. of cells/μl</td>
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<tr>
<td>Seropositive, FIV DNA positive (n = 19)</td>
<td>623 ± 123</td>
<td>19.2 ± 1.0</td>
<td>513 ± 97</td>
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<tr>
<td>Seronegative, FIV DNA positive (n = 10)</td>
<td>1,190 ± 261 (TM)</td>
<td>31.2 ± 5.1 (T)</td>
<td>671 ± 159</td>
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<td>Seronegative, uninfected (n = 7)</td>
<td>1,446 ± 243 (TM)</td>
<td>38.7 ± 2.3 (TM)</td>
<td>766 ± 149</td>
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</table>

* Data are means ± standard errors of the mean. Abbreviations: T, mean differs significantly (P < 0.05) from mean of FIV-seropositive cats by Student’s t test; TM, mean differs significantly (P < 0.05) from mean of FIV-seropositive cats by both Student’s t test and Mann-Whitney U test.

of CD4+ T lymphocytes and in the CD4+-to-CD8+ T-lymphocyte ratio (see Table 4) and depressed in vitro lymphocyte-proliferative responses to phytomogens (see Table 5).

The 20 naive cats housed with infected cats also remained asymptomatic for the duration of the study. One of these cats, cat no. 12 (group 5 [Table 1]), seroconverted (Fig. 1, lane 20) and became virus culture positive after being housed with FIV-seropositive cats for 8 months. This status was maintained for the remainder of the study. The remaining 19 cats remained seronegative by both IFA and Western blot analysis (Fig. 1, lanes 7 to 20). Viral cultures from these cats were negative for viral gag proteins and RT activity (Table 2).

Twelve of 17 pet cats in the Petaluma household were FIV seropositive at the start of the 4- to 5-year study and remained positive thereafter. A total of 6 of the 12 seropositive cats (Table 3) died of the AIDS-like syndrome associated with FIV infection, and 1 seronegative cat (cat P16) died of other causes during the same period. The remaining 10 cats were asymptomatic. Most of these cats were homeless prior to obtaining shelter in the Petaluma household. Many of them were feral. Therefore, they were at high risk of exposure to FIV infection and many were probably infected with FIV before they entered the home.

Detection of FIV DNA by PCR. FIV proviral DNA was detected in all 21 experimentally infected, seropositive cats (no. 6, 7, 9, 10, 17, 20, 21, 22, 26, 27, 32, 35, 36, 39, 2428, 2429, 2433, 2436, 2836, 2838, and 2839). Genomic DNA samples from both PBMC and bone marrow were positive for FIV gag- and env-specific sequences. Blood and bone marrow samples from the unexposed cats in groups 1 and 9 to 13 were uniformly negative. Of the 20 cats housed with experimentally infected cats, 11 were positive for FIV proviral DNA in either PBMC or bone marrow or both (Fig. 2; Table 2). In nine of these cats, both bone marrow and PBMC were positive, while in the remaining two cats, only bone marrow samples were positive for proviral DNA. Twelve seropositive cats in the Petaluma household and three of the five seronegative cats were positive for FIV proviral DNA in either PBMC or bone marrow or both (Table 3).

Detection of FIV-infected cells by in situ hybridization. ConA-stimulated PBMC cultures from seronegative, PCR-positive cats were examined by in situ hybridization with the 35S-labeled FIV DNA probe. Cultured PBMC from 4 of 10 seronegative, FIV genome-positive cats (no. 16, 19, 23, and 37) were positive for FIV RNA (Fig. 3D). A total of 2 to 5 cells per 500,000 cells were positive on days 3 to 6 post-ConA stimulation, while there were no positive cells detected in the PBMC cultures on day 19. PBMC cultures from four unexposed cats from groups 1 and 9 and cat no. 1, 2, 3, and 101 (Fig. 3C) and five unrelated SPF control cats (cat no. 106, 90458, 90468, 90490, and 90508) were negative for FIV RNA. By contrast, we found that the ConA-stimulated PBMC cultures from five experimentally infected cats (cat no. 7, 21, 26, 32, and 39) contained 1 to 2 detectable infected cells per 2,000 cells on day 3 and that the number of infected cells increased steadily thereafter (Fig. 3A and B). By day 6, there were 10 to 20 infected cells per 2,000 cells, and by day 13 there were 80 to 100 FIV-infected cells per 2,000 cells.

TABLE 5. Whole-blood lymphocyte stimulation by mitogens

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Seropositive, FIV DNA-positive cats (n = 15)</th>
<th>Seronegative, FIV DNA-positive cats (n = 9)</th>
<th>Seronegative, uninfected cats (n = 6)</th>
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<tbody>
<tr>
<td>Mean cpm of [3H]thymidine incorporation ± SEM</td>
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<tr>
<td>ConA</td>
<td>25,573 ± 4,900</td>
<td>51,460 ± 9,435 (TM)</td>
<td>54,817 ± 4,102 (TM)</td>
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<tr>
<td>ConA (adjusted)*</td>
<td>7.6 ± 1.0</td>
<td>14.8 ± 3.1 (T)</td>
<td>20.8 ± 3.4 (TM)</td>
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<tr>
<td>PWM</td>
<td>9,280 ± 1,449</td>
<td>17,592 ± 3,736 (TM)</td>
<td>15,319 ± 1,750 (TM)</td>
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<tr>
<td>PWM (adjusted)</td>
<td>2.8 ± 0.4</td>
<td>4.3 ± 0.1</td>
<td>5.7 ± 1.0 (TM)</td>
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<tr>
<td>No stimulation*</td>
<td>620 ± 36</td>
<td>759 ± 84</td>
<td>547 ± 88</td>
</tr>
</tbody>
</table>

* PWM, pokeweed mitogen; T, mean differs significantly (P < 0.05) from mean of FIV-seropositive cats by Student’s t test; TM, mean differs significantly (P < 0.05) from mean of FIV-seropositive cats by both Student’s t test and Mann-Whitney U test.

* Adjusted stimulation indicates mitogen counts per minute (cpm) divided by blood lymphocyte count.

* Mean background counts per minute (no mitogen present in culture).
and numbers of circulating CD4+ lymphocytes and in the CD4+-to-CD8+ cell ratios compared with unexposed as well as FIV genome-positive, seronegative cats, as determined by both Student's t and Mann-Whitney U tests.

**Whole-blood lymphocyte stimulation microassay.** There were no significant differences in mitogen-induced lymphocyte stimulation assays between FIV-unexposed cats and FIV genome-positive, seronegative cats (P > 0.05) (Table 5).

**FIV nucleic acids and antibodies in recipient cats.** To determine whether FIV genome-positive, seronegative cats could transmit viral genome, blood and bone marrow samples from four FIV-genome-positive, seronegative cats (cat no. 16, 19, 23, and 37) were transferred into the femoral bone marrow of four recipient cats (cat no. 90458, 90468, 90490, and 90508).

Cultured and uncultured PBMC from the recipient cats were negative for FIV nucleic acids in both PCR (Fig. 4; Table 6) and in situ hybridization assays (Fig. 3E) prior to the transfer. Uncultured PBMC samples from all four recipient cats were negative at 2 weeks posttransfer (Table 6). However, when the PBMC were cultured for 6 to 10 days following mitogenic stimulation, two of the four cats (cat no. 23 and 37) were positive only by PCR. At week 4, the same two cats were positive for FIV DNA in PBMC before culture, while after culture, all four PBMC samples were positive. By week 19, PBMC samples from three of the four cats were positive by PCR without in vitro culture (Table 6). The PBMC samples collected at weeks 6, 8, and 10 posttransfer were also positive for FIV nucleic acids by in situ hybridization following in vitro culture with ConA stimulation (Fig. 3F). About 10 to 1,000 cells per 500,000 PBMC tested positive by this assay.

Sera from the recipient cats were negative for FIV antibodies by IFA and Western blot analysis prior to the transfer study (Fig. 1, lanes 21 to 24) and consistently remained seronegative posttransfer (Fig. 1, lanes 25 to 28). In addition, none of the cats showed any outward signs of disease.

**Virus production following CD8+ T-cell depletion.** ConA-stimulated PBMC cultures from the four recipient cats did not produce FIV antigens by IFA or RT enzyme activity prior to or following the blood and bone marrow transfusion. This included cats that became FIV genome positive after the transfusion. In contrast, FIV was easily isolated from PBMC cultures of six experimentally FIV-infected seropositive cats.

It has been previously reported that CD8+ T cells suppressed HIV type I (HIV-1) replication in PBMC of healthy seropositive subjects. The depletion of CD8+ T cells from PBMC cultures of these human subjects and HIV-infected chimpanzees facilitated isolation of the virus (17, 15, 37, 38). A similar phenomenon in sooty mangabey monkeys was observed. Depletion of CD8+ T cells from PBMC cultures of seronegative animals resulted in the production of simian immunodeficiency virus (SIV) (12, 35). Because FIV was not isolated from PBMC or bone marrow of recipient cats, we explored the possibility that CD8+ T cells were suppressing FIV in the PBMC cultures. Following the CD8+ T-cell depletion, PBMC from donor (cat no. 16, 19, 23, and 37) and recipient (cat no. 90458, 90468, and 90490) cats were cultured in vitro. The supernatants from these cultures were negative for FIV antigens and RT activity. In contrast, PBMC samples from four experimentally FIV-infected seropositive cats tested positive for FIV antigens and RT enzyme activity within 2 to 3 weeks in culture.

**DISCUSSION**

Our data indicate that the viral genome-positive, seronegative state occurs following contact exposure to FIV and is partly similar to the previously described state of viral genome positivity and seronegativity in humans and monkeys for HIV and SIV, respectively (11–13, 20, 28, 29, 35, 36). The seronegative cats in our study had been housed with experimentally infected, seropositive cats for relatively long periods of time and had no prior evidence of FIV infection. Among a group of 20 FIV-naïve cats housed in rooms with infected animals, 1 animal developed conventional seropositive and virus-positive infection after an exposure period of 6 to 9 months, 8 cats remained negative for both viral genome and antibodies after 3 to 4 years, and 11 animals acquired the FIV genome without detectable levels of FIV antibodies at some point in the study. Because the PCR assay is known to give false-positive results, we examined genomic DNAs from PBMC of 28 SPF cats of the same bloodline for FIV DNA by PCR analysis prior to the exposure to FIV-infected cats. These cats were never exposed to FIV-infected cats and served as negative controls. Control cats remained negative for FIV DNA and antibodies. These results indicated that the SPF cats in our colony did not have endogenous FIV sequences detectable by PCR analysis. We also identified FIV genome-positive, seronegative cats in the Petaluma household from which FIV was first isolated, demonstrating that this phenomenon was not restricted to cats living in our laboratory environment. Three of the 5 seronegative cats housed with 12 naturally FIV-infected cats in the Petaluma household were positive for FIV genome. The overall percentages of cats which became FIV genome positive but seronegative after prolonged contact with FIV-seropositive cats at the University of California, Davis, and Petaluma were similar.

In HIV infection, host-mediated protective responses, such as CD8+ T-lymphocyte-mediated suppression, may contribute to suppressing viral gene expression, as has been reported elsewhere (14, 15, 37, 38). However, CD8+ T-cell depletion of PBMC from seropositive FIV DNA-positive cats failed to induce viral production in these cultures. Therefore, lack of FIV production in these cats cannot be simply explained by CD8+ T-cell-mediated suppression of viral replication.

The phenomenon of seronegative, viral genome-positive cats could be explained in several ways, one of them being delayed seroconversion. In some homosexual men, HIV-1 DNA was detected by PCR from 12 to 42 months before seroconversion (39). In an earlier experimental study, infected cats seroconverted within several weeks (42). However, a single cat took more than 14 months to seroconvert, even though it had isolatable virus in its blood at an early time. In a longitudinal study of an individual at high risk for HIV infection, positive proliferative response of T helper
cells to HIV env indicated exposure to HIV infection almost 1 year before evidence of viral infection by PCR and serology (8). It is therefore possible that a number of people who have been exposed to the virus may remain in the seronegative state for a lengthy period of time. We have monitored the FIV genome-positive, seronegative cats for over a year, and there has been no change in their serologic status. The 1 cat (cat no. 12) that seroconverted while in contact with infected cats has remained seropositive; none of the remaining 19 cats has seroconverted.

The second possibility is that genome-positive, seronegative cats have acquired the FIV genome through either a latent or defective virus infection. In vitro cell culture studies have shown that human monocytic and lymphocytic cell lines nonproductively harboring the HIV genome generate spliced HIV transcripts (30). Stimulation with agents that activate the HIV-1 long terminal repeat results in production of an increasing amount of small mRNA, unspliced RNA species and infectious virus (30). Zack and colleagues have shown that HIV-1 can enter nonproliferating quiescent human peripheral blood lymphocytes and initiate viral synthesis at the same level as that in mitogen-stimulated cells but that quiescent cells are unable to synthesize complete viral DNA (43). Therefore, it seems that latent HIV infection is characterized by incomplete viral genome synthesis and an absence of infectious virus. We also observed the transient appearance of viral RNA transcripts in mitogen-activated PBMC from 4 of 11 FIV genome-positive, seronegative cats but were unable to demonstrate production of RT enzyme or FIV core proteins in these cultures. This was in sharp contrast to findings with ConA-stimulated PBMC cultures from FIV-seropositive cats, which produced progressively more viral transcripts with time and elaborated both RT and FIV p24 antigen. Because neither ConA stimulation of PBMC nor the CD8+ T-cell depletion could induce synthesis of infectious FIV in these cats, our data indicate that the FIV genome in seronegative, genome-positive cats was defective. Infective blood and saliva from viremic cats may contain a swarm of FIV variants, and some of these variants could be infectious but replication defective. Such hypothetical FIV variants may fail to establish a conventional disease course or host immune response.

The third possibility is that FIV genome-positive-, seronegative cats were infected with FIV in such a way as to cause an atypical immune response and disease course. It has been shown that conventional FIV infection and disease follow either direct parenteral infection of the virus or bites via infective saliva (41, 42). Although considered uncommon, postpartum transmission of HIV through exposure of infants to infected breast milk might represent an analogous means of oral spread (17, 21, 32, 44). Experimentally administered bites lead to viremia and rapid seroconversion in cats (41). However, biting was generally not observed among cats in our experimental groups, and it is conceivable that cats housed with infected animals may have been exposed to minuscule doses of virus following ingestion of infective saliva, feces, and urine in the process of mutual grooming or using the same food and water dishes. Virus entry could also be facilitated by the presence of minor abrasions in the oral mucosa. Such virus would enter the body through the mucous membranes, an atypical route of infection. This might in turn result in strong immediate local immunity and rapid containment of the viral genome in cells. If virus replication was very low prior to containment, the antigenic stimuli may have been insufficient to stimulate antibodies. The route of transmission (intravenous versus mucosal), degree of virus load, and nature of virus variants may play key roles in determining the course of the infection and seroconversion.

The clinical significance of seronegative, viral genome-positive latent infections in humans and cats remains undetermined. Critical information is needed to evaluate further the overall rate of seroconversion among seronegative, HIV genome-positive individuals. There is uncertainty over the median time period of HIV infection prior to seroconversion. Likewise, it is uncertain whether such individuals can transmit the virus to other susceptible people. At present, there is evidence for the occurrence of seronegative and viral genome-positive infections in sooty mangabey monkeys naturally infected with their endemic strain of SIV (35). When blood from seronegative but PCR-positive sooty mangabeys was transferred to naive animals, recipients became seropositive and the presence of SIV DNA could be detected (12).

Our study demonstrates that following the transfer of blood and bone marrow from seronegative, FIV genome-positive cats, three of the four naive cats acquired FIV DNA in their PBMC. Whether these cats will ever show active virus production remains to be seen. It is also possible that over time, FIV reactivity may be lost if a productive viral infection is not established. Early host immune responses to FIV infection remain undetermined. The FIV model should prove invaluable to understand host-mediated suppression of virus production in the initial stages of infection and its relation to latency and delayed seroconversion.

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