Development and Evaluation of Immunoassay for Detection of Antibodies to the Feline T-Lymphotropic Lentivirus (Feline Immunodeficiency Virus)

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The feline T-cell lymphotropic lentivirus (feline immunodeficiency virus) is a recently described feline-specific retrovirus that can produce chronic immunodeficiency-like disorders in cats. A microloduction plate format enzyme-linked immunosorbent assay has been developed to detect the presence of antibody to the virus in feline serum or plasma. Temporal studies performed with experimentally infected animals show that seroconversion can be demonstrated 3 to 4 weeks after exposure to the virus. Results of a serosurvey (n = 1,556 samples) indicate that infection is fairly common in both clinic (5.2%) and sick cat (15.2%) populations. Western blot (immunoblot) and sodium dodecyl sulfate radioimmunoprecipitation assays were developed to confirm microloduction plate test results and to identify peptides specific for the feline immunodeficiency virus. All microloduction plate test positive results and selected negative results were confirmed by one or both of these procedures. These data demonstrate that this microassay plate enzyme-linked immunosorbent assay is a very sensitive and specific test for detection of antibody to the feline immunodeficiency virus.

A recently identified retrovirus has been shown to be the causative agent of a chronic immunodeficiency-like syndrome in cats (21). In initial reports the virus was named feline T-lymphotropic lentivirus to reflect the tropism of the virus for feline T cells and the classification of the agent as a member of the lentivirus subfamily of retroviruses (12, 22). The name of the virus has been recently changed to feline immunodeficiency virus (FIV) to conform with international nomenclature for immunodeficiency-linked viruses (24). The FIV agent has a strong but not absolute tropism for the feline T-lymphocyte cell line, which may be responsible for the immunosuppressive nature of the virus. Viral particle morphology and the Mg++ metal requirement of the viral reverse transcriptase are supportive of the classification of the virus as a lentivirus.

The virus is distinct from previously described feline retroviruses and represents the first report of a feline-specific lentivirus (21). Members of the lentivirus subfamily infecting other species include the human immunodeficiency virus (HIV) (8, 15), visna virus in sheep (19), caprine arthritis-encephalitis virus (7), equine infectious anemia virus (20), bovine immunodeficiency virus (9), and simian immunodeficiency virus (3).

The clinical course of FIV infection appears to be characterized by a lengthy asymptomatic phase persisting several months or perhaps years, during which viral infection can be demonstrated but clinical symptoms are not apparent (24). This period of apparent viral latency is typical of lentiviral infections and frequently precedes the development of clinical abnormalities (10). Clinical symptoms most commonly associated with FIV infection include rhinitis and gingivitis, anemia, diarrhea, pustular dermatitis, and generalized lymphadenopathy (12, 22). Symptoms vary among infected animals but are characterized by a chronic and persistent nature.

The FIV is infectious within feline populations and can be transmitted after intimate and prolonged contact. Initial reports suggest that biting may be an important mode of viral transmission (24). Isolates of FIV have been identified in the United States (21), the United Kingdom (11), and Japan (13). A limited serosurvey in the United States reported that 1 in 18 healthy cats and 10 of 25 unhealthy cats were seropositive for FIV infection (21).

The continuous propagation of FIV in an established Crandell feline cell line has permitted the isolation and purification of large quantities of virus. The highly purified virus was used to develop a sensitive microloduction plate-based enzyme-linked immunosorbent assay (ELISA) for detection of feline antibody specific for the FIV agent. In this report we describe the development of the FIV microloduction plate ELISA, partially characterize immunoreactive viral peptides, and present data on the prevalence of viral infection in several populations (n = 1,556). The results of this survey demonstrate that FIV infection is relatively common and widespread in the United States.

MATERIALS AND METHODS

Virus and cell culture. The FIV was propagated in chronically infected Crandell feline kidney (Cfrk) cells (6). The virus was concentrated from tissue culture fluids by precipitation with polyethylene glycol (4) and purified by density gradient centrifugation on glycerol gradients as previously described (18).

The FIV microloduction plates were prepared by coating microloduction wells with 100 μl of an inactivated detergent-disrupted preparation of FIV antigen (5.0 μg/ml). Samples of feline serum or plasma were diluted 1:100 in 20 mM sodium phosphate–150 mM NaCl (pH 7.4) containing calf serum and bovine serum albumin. Diluted samples (100 μl) were ap...

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plied to individual microdilution wells and incubated for 30 min at room temperature. The contents of the microdilution plates were aspirated, and the plates were washed (five times) and incubated (30 min, room temperature) with goat anti-feline horseradish peroxidase conjugate (Kirkegaard and Perry). The contents of the microdilution plates were aspirated, the plates were washed (five times), and substrate solution (100 µl) was added. The substrate solution was prepared immediately before the assay by mixing equivalent portions of 0.1% 3,3',5,5'-tetramethylbenzidine in 60% methanol–40% glycerol with 0.04% hydrogen peroxide in 0.1 M dibasic potassium phosphate-citrate (pH 4.8) containing 0.01% Thimerosal. The enzymatic reaction was carried out for 15 min at room temperature and then stopped by addition of 100 µl of dilute hydrofluoric acid solution (1:400). Individual microdilution well optical density values were determined spectrophotometrically at 650 nm.

To minimize interassay variability, ELISA optical density values were normalized by using positive and negative control reagents assayed on each microdilution plate. The presence or absence of antibody to FIV was determined by relating the A650 of the sample to the A650 of the positive control reagent. The positive control contains a standardized level of antibody to FIV in feline serum. The relative level of FIV-specific antibody in the sample was determined by calculating the sample-to-positive (S/P) ratio in the following manner: (sample A650 – negative control A650)/(positive control A650 – negative control A650). Samples with S/P ratios less than 0.5 are classified negative. S/P ratios equal to or greater than 0.5 are positive for antibody to FIV.

**Clinical samples.** The FIV ELISA was used to screen a total of 1,556 samples obtained from five sources: 223 from the Boston Refugee League (Boston, Mass.), 130 from the Chicago Cat Clinic (Chicago, Ill.), 459 from Clinipath Laboratories (Valparaiso, Ind.), 145 from the Veterinary Reference Laboratory (Dallas, Tex.), and 599 from the University of California (San Diego). Samples from the Boston Refugee League, Chicago Cat Clinic, Clinipath, and the Veterinary Reference Laboratory were not selected on the basis of previous disease status and represent a collection of samples received by these clinics and laboratories. Samples obtained from the University of California were obtained from animals with a previous unspecified disease or illness. This set of samples should be regarded as comprising a sick cat population.

Samples for the seroconversion studies were obtained from the Cornell Feline Health Center (Ithaca, N.Y.) and the University of California (Davis). These were obtained from specific-pathogen-free animals after inoculation with either infectious cell-free tissue culture fluid (1 ml), FIV-infected whole blood (1 ml), or 0.44-μm-filtered plasma (2 ml) from infected animals.

**Western blot assay.** The Western blot (immunoblot) protocol used was a modification of the procedure initially described by Towbin et al. (23). Purified FIV was disrupted with sodium dodecyl sulfate (SDS) and mercaptoethanol and separated with a 10% SDS-polyacrylamide gel. Viral proteins were transferred to nitrocellulose sheets, which were then blocked with detergent and calf serum. Individual strips were cut from each sheet and incubated for 2 h with a 1:100 dilution of sample prepared in a phosphate-buffered saline solution containing 0.05% Tween 20, 1% bovine serum albumin, and 30% calf serum. Strips were washed exhaustively with phosphate-buffered saline–0.05% Tween 20 and incubated for 1 h with goat anti-feline horseradish peroxidase conjugate. The wash cycle was repeated, and the precipitating substrate 4-chloronaphthol was added. Reactions were stopped by washing with deionized H2O, and results were immediately interpreted. The presence of two or more FIV viral bands was necessary to confirm a sample as positive for antibody to FIV. The molecular weights of FIV-reactive peptides were determined by comparison with immunoperoxidase-stained Western blot strips with Coomasie blue-stained polyacrylamide gels and amido black-stained nitrocellulose strips (R. Steinman, T. O’Connor, Q. Tonelli, K. Lawrence, C. Seymour, J. Goodness, N. Pedersen, and P. R. Andersen, submitted for publication). Uninfected Crk host cell extract was used to prepare Western blot strips to identify reactive bands unrelated to FIV infection. These were used in the identification of nonspecific bands appearing on FIV blot strips.

**RIPA-SDS-PAGE.** For the radioimmunoaassay (RIPA) with SDS-polyacrylamide gel electrophoresis (PAGE), the FIV productively growing in cell culture was metabolically labeled with [35S]methionine and [35S]cysteine at 37°C for 4 h. Cells were lysed in 10 mM phosphate buffer (pH 7.5) containing 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, and 100 Kallikrein inactivator units of aprotilin per ml (lysis buffer). Cell lysates were clarified by centrifugation at 100,000 × g for 30 min before use. Generally, 100 µl of viral extract was mixed with 5 µl of serum sample and incubated for 18 h at 3°C. Samples were concentrated by adding 0.2 ml of a 5% suspension of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) in 10 mM phosphate (pH 7.5)–100 mM NaCl–1% Triton X-100–0.1% SDS and centrifuging. The pellet was washed three times in lysis buffer, heated in sample buffer, and applied to a 12% SDS–polyacrylamide gel (14). Gels were processed for fluorography and exposed at −70°C to Kodak XAR-5 film.

**RESULTS**

FIV ELISA detection of seroconversion in response to experimental infection. Serum samples from a total of 14 experimentally infected cats were obtained to assess the sensitivity of the microdilution plate test with respect to seroconversion. Temporal samples were obtained for each animal and assayed by the FIV ELISA. Western blots and/or RIPA-SDS-PAGE were used to confirm both positive and negative results.

Seroconversion was demonstrated in all animals by 4 weeks after the initial exposure. Microdilution plate data for cats 2429 and 2838 are shown in Fig. 1 and are typical of the entire series of experimentally infected animals. Western blots and RIPA-SDS-PAGE confirmatory test data are shown in Fig. 2 through 5 and demonstrate seroconversion paralleling the microdilution plate test results. With the exception of one cat, which died during the study (week 14), all cats remained seropositive during the testing period.

Most of the experimentally infected animals showed similar clinical manifestations, including an initial neutropenia lasting 4 to 5 weeks, fever lasting several days postinfection, and a generalized enlargement of peripheral lymph nodes occurring 4 weeks postinfection and persisting for 2 to 9 months. However, with the exception of the single cat cited above, none of the experimentally infected animals developed symptoms associated with late-stage FIV infections (24).

The Western blot assay of the two temporal series showed initial reaction to the FIV gag proteins p15 and p26 (Fig. 2 and 3). Reaction to additional FIV proteins (p10, p32, gp40,
p47, and p65) occurred during the course of infection (Steinman et al., submitted). Serum samples from specific-pathogen-free cats with experimentally induced FIV infection were not reactive on immunoblots of HIV, HIV type 2, simian immunodeficiency virus, caprine arthritis-encephalitis virus, or feline leukemia virus (24). The RIPA–SDS-PAGE assay showed positive results after approximately the same time period as the microdilution plate test and Western blot assay (Fig. 4 and 5). FIV proteins immunoprecipitated by the temporal samples were identified as gp130, p110, p47, gp40, p36, and p22 (Steinman et al., submitted).

**FIV ELISA: clinical evaluation.** The frequency distributions for the clinic and sick cat populations are shown in Fig. 6 and 7, respectively. Results are plotted as frequency versus the S/P ratio. As described in Materials and Methods, an S/P ratio greater than 0.5 indicates that the sample contains significant levels of antibody to FIV. The seropositive rate was 5.2% for the clinic population (n = 957) and 15.2% for the sick cat population (n = 599).

**Western blot and/or RIPA–SDS-PAGE confirmatory assays were conducted on all samples that tested positive by the microdilution plate test. Each of these samples was positive by the confirmatory procedures. Western blot assays of FIV antibody-positive samples typically showed a strong reaction to the FIV gag proteins p26 and p15 and variable reactivity to additional FIV proteins (10,000, 32,000, 40,000, 47,000, and 65,000 daltons) (Steinman et al., submitted). Staining of two or more FIV peptides was required for positive result confirmation by Western blotting, whereas reaction to gp130 was required for confirmation by RIPA–SDS-PAGE. The RIPA–SDS-PAGE assays of positive samples demonstrated variable reactions to proteins of 130,000, 110,000, 47,000, 40,000, 36,000, 22,000, and 15,000 daltons; however, all plate test-positive samples immunoprecipitated gp130. A small percentage (3%) of microdilution plate test-positive samples were reactive to only a single FIV peptide (p26 or p15) in the Western blot assay. These samples were positive when tested using the RIPA–SDS-PAGE assay. A set of 150 plate test-negative samples were negative when tested by the Western blot assay.
DISCUSSION

The seroconversion studies demonstrate that a significant antibody level to FIV can be measured 3 to 4 weeks after initial exposure to the virus. The long time course (13 to 14 months) of continued antibody response shown for cats 2429 and 2838 demonstrate the persistent nature of infection. The extended disease-free period observed for 13 of 14 experimentally infected cats may reflect the long incubation period of the virus. A similar disease-free state has been well documented after seroconversion to HIV in humans (16).

Western blot assays show the initial reaction to the FIV gag proteins (primarily p15 and p26), whereas the RIPA-SDS-PAGE technique showed an initial reaction to the FIV env proteins (gp130, p110, gp40). Reaction to additional viral proteins was observed during the course of infection with both techniques. This pattern of antibody response, and the differing capacity of the Western blot and the RIPA-SDS-PAGE techniques to detect sets of virus-specific antibodies, has been observed in HIV antibody studies in humans (1, 2, 5). The naturally occurring antibody response to FIV in cats may serve as a useful model for the similar response to HIV in humans.

The FIV microdilution plate test can be evaluated by examining the distribution of assay results for a collection of positive and negative samples relative to the assay cutoff. ELISA plate test data have been reported for a total of 1,556 samples. The mean S/P ratio was 0.019 (standard deviation, 0.048) for the combined negative population and 2.46 (standard deviation, 1.18) for the overall positive population. The assay cutoff value (S/P ratio, 0.5) is positioned 0.481 units or 10.02 standard deviations above the mean of the S/P ratio for the negative samples. Similarly, the assay cutoff is below assay results for all 141 positive samples tested and substantially below the mean S/P ratio for the positive population. None of the samples included in this study was incorrectly identified by the microdilution plate test when assayed by
the Western blot or RIPA-SDS-PAGE procedures. The relatively large standard deviation in S/P ratio observed for the positive population reflects variation in antibody response in individual animals.

The results of this serosurvey indicate that FIV infection is widespread in both the clinic (5.2%) and the sick cat (15.2%) populations. The substantially higher incidence of viral infection in the sick cat population apparently reflects the disease-causing potential of the virus. The overall seropositive rate is similar to the feline leukemia virus seropositive rate reported for similar populations (17). The identification of FIV as a transmissible disease-causing agent and the demonstration of the extent of infection in cat populations has serious implications in veterinary medicine. The seropositive rate found for both the clinic and the sick cat populations suggests that infection by FIV will provide a meaningful characterization for previously undiagnosed disease syndromes in cats. If the potential of the virus to cause disease is as high as reported, the benefit of FIV screening for veterinary clinicians would be substantial.

LITERATURE CITED
