Humoral immune response to feline immunodeficiency virus in cats with experimentally induced and naturally acquired infections

I. F. Egberink, DVM, PhD; C. E. J. M. Keldermans; M. J. M. Koolen, PhD; M. C. Horzinek, DVM, PhD

SUMMARY

Sera from cats with naturally acquired and experimentally induced feline immunodeficiency virus (FIV) infections were tested by immunoblot analysis, radioimmunoprecipitation assay (RIPA), and a complex trapping/blocking ELISA. In sequentially obtained samples from experimentally inoculated cats, antibodies against the envelope protein gp120 and the core protein p15 were the first to appear, as indicated by results of RIPA, using lysates of FIV-infected lymphocytes. Antibodies could be detected as early as 2 weeks after infection, followed by a response against p24, p43, and p50. By immunoblot analysis, p24 and p15 were the first proteins detectable between postinoculation weeks 3 and 5; an anti-envelope response was never found by use of this assay, but was found by RIPA. Using the latter test, most sera of naturally infected cats were found to recognize the major core protein p24 in addition to 1 or more minor core proteins. All 40 sera tested precipitated the envelope protein; 3 reacted exclusively with it. A complex trapping/blocking ELISA was developed to quantitate the anti-p24 response. Sera from healthy FIV-infected cats were shown to have higher anti-p24 titer than did those from diseased cats.

Feline immunodeficiency virus (FIV), a lentivirus, was first isolated in 1986.[1] The virus has been found in cats throughout the world and is an important pathogen. Frequently observed clinical signs of FIV infection are emaciation, weight loss, lymphadenopathy, chronic stomatitis/gingivitis, chronic diarrhea, and dermatitis.[2,3] Also, FIV infection in cats is becoming an important animal model for human immunodeficiency virus (HIV) infection. The virus resembles HIV in biological and physical aspects[1,4]; both infections are associated with an asymptomatic phase of months or even years before clinical signs develop.[5] Immunologic abnormalities, similar to those in HIV-infected people, have been described in cats.[6,7]

Diagnosis of FIV infection is based on virus isolation from blood leukocytes and results of serologic testing.[1] On a routine basis, antibody detection by the immunofluorescent antibody (IFA) assay, ELISA, or immunoblot analysis can be performed; immunoblotting is mostly used as a confirmatory test. When purified virus is used as antigen, it preferentially detects anticore antibodies;[4,8] an antienvelope response is only incidentally detected. For HIV infection, it was shown that the radioimmunoprecipitation assay (RIPA) is the better test to detect antibodies directed against envelope antigens.[9] However, using lysates of FIV-infected cells, Hosie et al[10] were able to also detect an antienvelope response by immunoblotting.

For a rational selection of antigens to be used as serodiagnostic reagents (eg, in second-generation assays based on products obtained by recombinant DNA technology), an understanding of the antibody response after FIV infection is necessary. With this objective in mind, we studied the reaction pattern of sera from experimentally inoculated and field-infected cats by use of immunoblotting and RIPA. To assess presence and amount of p24-specific antibodies, a complex trapping/blocking (CTB) ELISA was developed, using monoclonal antibodies (MAB) recognizing 2 epitopes. Sera of sick and healthy FIV-infected cats were tested to look for quantitative differences that could give prognostic clues. In people in the later stages of acquired immune deficiency syndrome, antibodies to core proteins seem to diminish and are less consistently found.[11]

Materials and Methods

Virus—The Petaluma strain of FIV[a] was obtained. Another strain termed FIV-113 had been isolated from a FIV-infected cat in The Netherlands. Virus was grown either in blood mononuclear cells or in thymocytes from specific-pathogen-free donor cats. Cells were stimulated with concanavalin A (5 μg/ml) and grown in RPMI 1640 medium with 10% fetal bovine serum and 100 U of recombinant human interleukin 2/ml; alternatively, Crandell feline kidney (CRFK) cells were used for growing virus.

Experimentally induced infection—Two cats were inoculated with the Petaluma strain of FIV by intraperitoneal and SC administration—1 cat with culture supernatant of infected lymphocytes, the other cat with homologous FIV-infected lymphocytes. Once a week, a blood sample was collected for virus isolation, serologic testing, and determination of some blood variables (hematocrit, leukocytes, and differential WBC count). Once a week, reverse transcriptase activity[1] was measured in the supernatants of the lymphocyte cultures, which were monitored for at least 56 days.

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From the Institute of Virology, Department of Infectious Diseases and Immunology, School of Veterinary Medicine, State University of Utrecht, Yalelaan 1, 3584 CL Utrecht, The Netherlands, and Organon Teknika BV, Boxtel, The Netherlands (Koolen).

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[a] Courtesy of Dr. Niels C. Pedersen, University of California, Davis.

Antibody Detection Assays

Antibodies against FIV were measured by use of an IFA test, and FIV-positive sera (n = 76) were selected. All sera were tested by use of immunoblotting, 40 sera by RIPA and 60 by CTB-ELISA.

Immunofluorescent antibody test—For the IFA test, sera were assayed at a dilution of 1:20 on FIV-infected CRFK cells as described.[2]

Immunoblotting—The procedure for immunoblotting was essentially the same as described.[12] Gradient-purified FIV from persistently infected CRFK cells was separated by use of 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose sheets[b] (pore diameter 0.45 μm), using an semidry-blotting apparatus.[c] After transfer to nitrocellulose, the sheets were cut into strips of 3 mm and blocked for 2 hours at 37 C in phosphate-buffered saline solution containing 0.1% Triton X-100 and 0.05% pig skin gelatin (gelatin buffer). Sera were diluted 1:50 in gelatin buffer and incubated for 1.5 hour at 20 to 22 C. Strips were washed in gelatin buffer, and goat anti-cat horseradish peroxidase-conjugated immunoglobulins were added. After another hour of incubation and subsequent washing of the strips, 4-chloro-1-naphtol was added as a chromogen. The reaction was stopped by rinsing the slides in water, and the results were subsequently interpreted.

Radioimmunoprecipitation assay—Labeling of FIV-infected cells and the RIPA procedure were performed essentially as described.[12] Briefly, thymocyte cultures 5 days after infection with FIV or persistently infected CRFK cells were labeled overnight (16 hours) with [^{35}S] methionine. Lysates of the cells were clarified at 10,000 × g for 30 minutes and incubated with 5 μl of serum in tris EDTA saline buffer (TESV) containing 0.5% Triton X-100 and 2 m*M* phenylmethylsulfonylfluoride at 4 C for 16 hours. Subsequently, immune complexes were precipitated with formaldehyde-fixed *Staphylococcus aureus* cells[d] for 45 minutes at 4 C. The precipitates were washed 3 times with TESV plus 0.1% Triton X-100, and were finally resuspended in Laemmli sample buffer. Proteins were analyzed by SDS-PAGE and visualized by fluorography.

Complex trapping/blocking ELISA—A CTB-ELISA[13] was developed. Monoclonal antibodies used in this assay were produced according to standard procedures. Mice were inoculated with gradient-purified FIV mixed with an equal amount of complete Freund's adjuvant. Booster inoculations with the same antigen preparation in incomplete adjuvant were given to mice 3 weeks later. Mouse spleen cells were fused with P3×63-Ag8-653 mouse myeloma cells 3 days after final booster administration. Hybridomas were grown in hypoxanthine-aminopterine-thymidine medium and screening was performed, using FIV-coated microtitration plates. From the selected and cloned hybridomas, ascitic fluid was prepared in pristane-pretreated mice. Antibodies were purified by sodium sulfate precipitation and were biotin-labeled. Two MAB (5E6D11 and 11C7C7) recognizing different epitopes on the major core protein as detected in competition assays (results not shown), were used.

Microtitration plates[e] were coated overnight at 4 C with a 1:2,000 dilution of MAB 5E6D11. Plates were washed with phosphate-buffered saline solution containing 0.05% Tween 20 (washing solution) and blocked with 200 μl of 5% newborn calf serum in washing solution (ELISA buffer) for 1 hour at 37 C. The FIV antigen was disrupted in 0.1% Triton X-100 and diluted in ELISA buffer containing 0.1% Triton X-100. Virus antigen and serum were mixed in a separate microtitration plate, and the mixture (total volume, 100 μl) was added to the MAB-coated plate. Sera were tested at twofold dilutions starting at 1:20. After

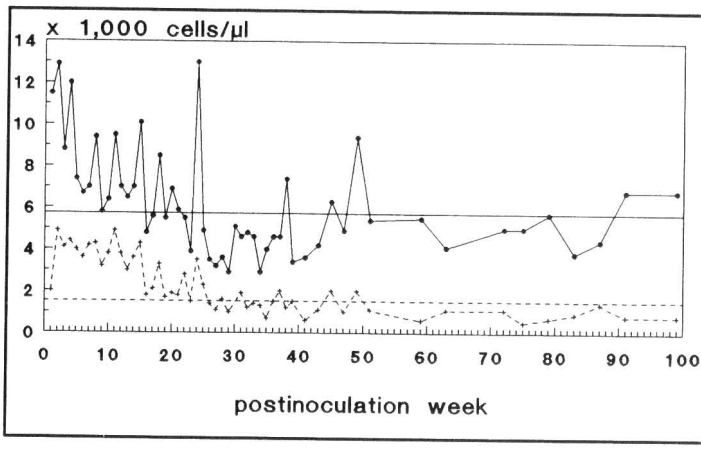


Figure 1—Total leukocyte (●) and lymphocyte (+) counts in sequentially obtained samples from cat 201 after experimentally induced infection with the Petaluma strain of feline immunodeficiency virus (FIV). Number of cells is expressed as 1,000 cells/μl. The solid line indicates the minimal normal value for leukocytes (5.7 × 10^3 cells/μl) and the dashed line indicates the minimal value for lymphocytes (1.5 × 10^3 cells/μl).

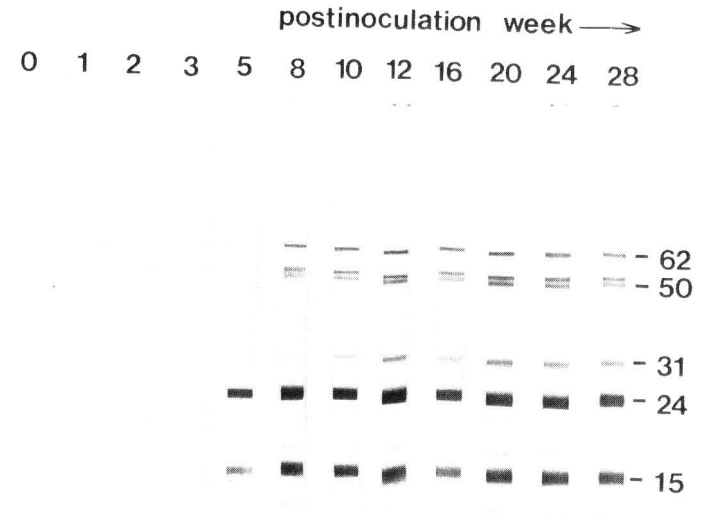


Figure 2—Immunoblot analysis of FIV, reacted with sequentially obtained serum samples from experimentally infected cat 201 (Fig 1). Numbers to the right indicate apparent molecular mass (kilodaltons) of the viral proteins. Numbers of the lanes represent the weeks after inoculation.

[b] Schleicher & Schuell GmbH, Dassel, Germany.
[c] LKB Producter AB, Bromma, Sweden.
[d] Pansorbin, Calbiochem, San Diego, Calif.
[e] Greiner high binding, Alphen a/d Rijn, The Netherlands.

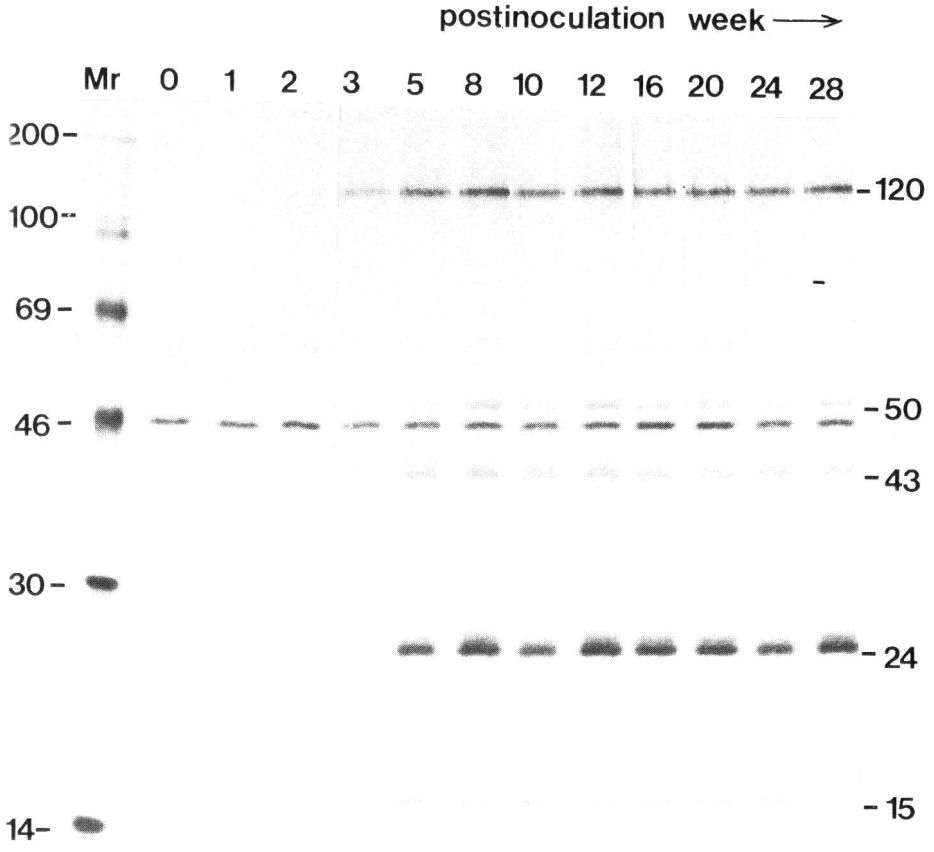


Figure 3—Gel electrophoresis of immunoprecipitates from lysates of [^{35}S]methionine-labeled FIV-infected lymphocytes and sequentially obtained serum samples from cat 201 (Fig 1). Numbers to the right refer to the molecular mass (kilodaltons) of the viral proteins. To the left, molecular mass of the [^{14}C] marker proteins is shown.

hour of incubation, biotinylated MAB 11C7C7 was added, followed by a 1:4,000 dilution of streptavidine peroxidase[f] in ELISA buffer. The proper antigen concentration and dilution of the biotinylated MAB were chosen from dose-response curves determined on MAB 5E6D11-coated plates. After washing the plates, the substrate tetramethyl benzidine[f] was added. The reaction was stopped by adding 2*N* H_2SO_4, and absorption was measured at 450 nm.

Results

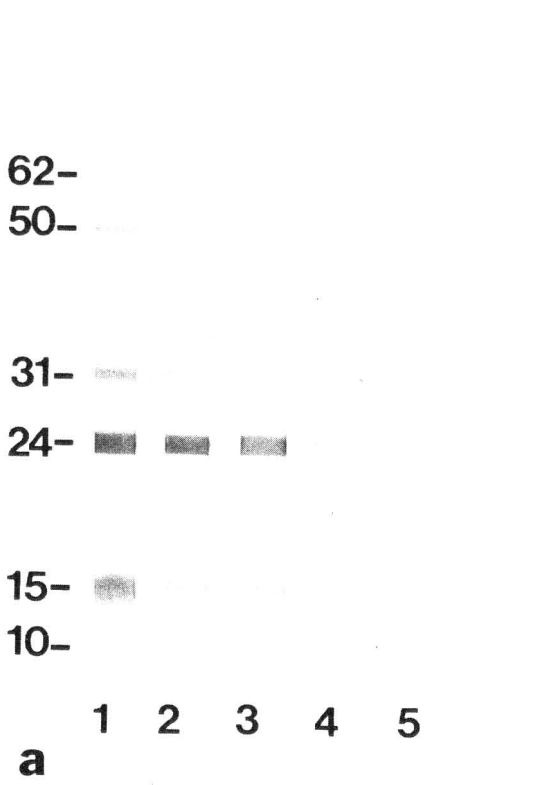
Experimentally induced infection—In addition to 4 days of fever, starting 4 weeks after inoculation in one of the infected cats, clinical signs of FIV infection were not observed during the entire period of the study (> 2 years). Virus was isolated for the first time during postinoculation week (PIW) 4. Starting 4 to 6 months after inoculation, both cats developed progressive leukopenia, mainly attributable to lymphopenia (Fig 1). Neutrophil numbers were only incidentally below the normal range. Leukocyte values were consistently in the low to low-normal range. Lymphocyte counts had some fluctuations, but gradually decreased to <1,500 cells/μl during the first year and to < 800 cells/μl during the second year of infection, where they remained.

Cats became seropositive (by IFA testing) between PIW 3 and 4. By immunoblotting, antibodies against p24 and p15 were first detected (PIW 3 to 5), followed by antibodies against p10 (PIW 8, Fig 2). At that time, antibodies against proteins of 31, 50, and 62 kDa were recognized. A reaction with the env protein (gp 120) was never observed by immunoblotting. With respect to this protein, RIPA was more sensitive: as early as PIW 2, minor reaction against p15 and gp120 could be observed, which was earlier than recognition of p24 and p43 (Fig 3).

Field infection—In 40 sera from field cats selected on the basis of distinctly or weakly positive IFA test results, the patterns observed by RIPA or immunoblotting were different (Fig 4). By immunoblotting, most cats had distinct response against p24 and p15. Additional bands of 10, 31, and 62 kDa were found; the 43-kDa transmembrane protein was seldom visible. In the 50-kDa region, a double band was conspicuous. Using RIPA, however, reactions against the env protein are also detectable; gp100, with its precursor gp130, is precipitated in addition to p50, p43, p24, and p15.

Cats differ in reactivity to p24. Some field sera recognized p24 only poorly or not at all (Fig 4A lanes 4 and 5); these were also weakly positive by IFA testing. The reactions were not false-positive results, however, as must be concluded from results of the RIPA performed in parallel (Fig 4B). All 40 field sera precipitated gp 100, the envelope protein, and its 130-kDa precursor, and the variable reactivity to the major gag protein p24 was again observed. Serum 4 precipitated only small amounts of p24

[f] Sigma Chemical Co, St Louis, Mo.



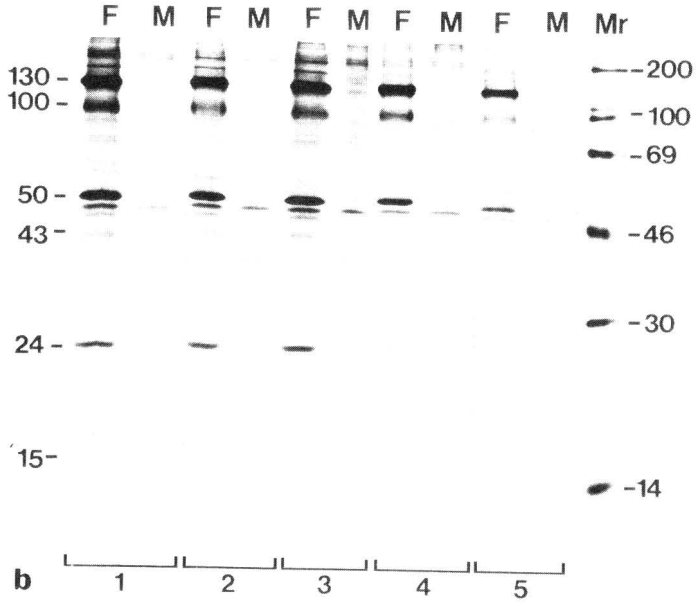


Figure 4—Sera of FIV-infected cats tested by immunoblotting with gradient-purified FIV (a) and by radioimmunoprecipitation (b) of [^{35}S]methionine-labeled FIV (lanes F)- and mock (lanes M)-infected Crandell feline kidney cell lysates. Results of 5 sera (1–5) are shown. To the left, molecular mass of the viral proteins is indicated (kilodaltons), lane Mr indicates the [^{14}C] marker proteins with their molecular mass (kilodaltons) indicated to the right.

in the presence of a strong reaction against both env proteins (Fig 4B). The p50 gag precursor protein also was precipitated. Serum 5 did not recognize p24 at all, but reacted with its p50 precursor. The RIPA was performed on 2 sequentially obtained samples (2 and 3) from the same cat (Fig 5). Serum 2 was obtained at the time that

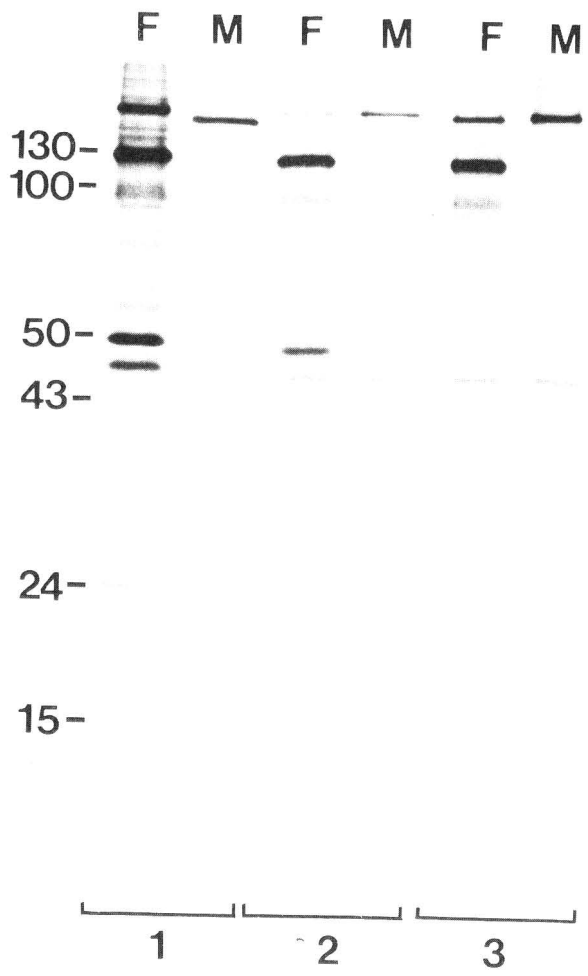


Figure 5—Radioimmunoprecipitation of [^{35}S]methionine-labeled FIV (lanes F)- and mock (lanes M)-infected cell lysates, with 3 sera. Serum 1 is a positive-control serum from an experimentally infected cat, sera 2 and 3 are from a field case of FIV infection and were obtained 6 weeks apart. To the left, molecular mass (kilodaltons) of the virus-specific proteins is shown

FIV diagnosis had been made by results of IFA testing. Serum 3 was obtained 6 weeks later, at the time the cat was euthanatized because of severe opportunistic infections. By immunoblotting of serum 2, only a minor reaction against p24 and p15 could be seen, whereas serum 3 did not recognize the major core protein at all (results not shown). Using RIPA, serum 2 precipitated the precursor protein p50, but not p24. Interestingly, serum 3 had distinct reaction only with the envelope proteins, gp100 and the 130-kDa protein. In neither serum could reaction with the transmembrane protein p43 be detected. We found 3 sera of 40 that reacted only with the envelope protein.

Results of the CTB-ELISA correlated well to results of immunoblotting and the RIPA. A wide range of titer, from 20 to > 2,560, was determined. Sera that did not recognize p24 by immunoblotting also were negative (< 20) in the CTB-ELISA. Titer for 60 serum samples from cats for which the health status was known was determined (Fig 6). All sick cats (n = 43) had some chronic opportunistic infection. Samples from healthy seropositive cats (n = 17) were collected during routine testing or from healthy housemates of FIV-positive cats. In healthy cats, significantly (Mann Whitney test[14]: $P = 0.000$) higher titer was detected, compared with that in sick cats.

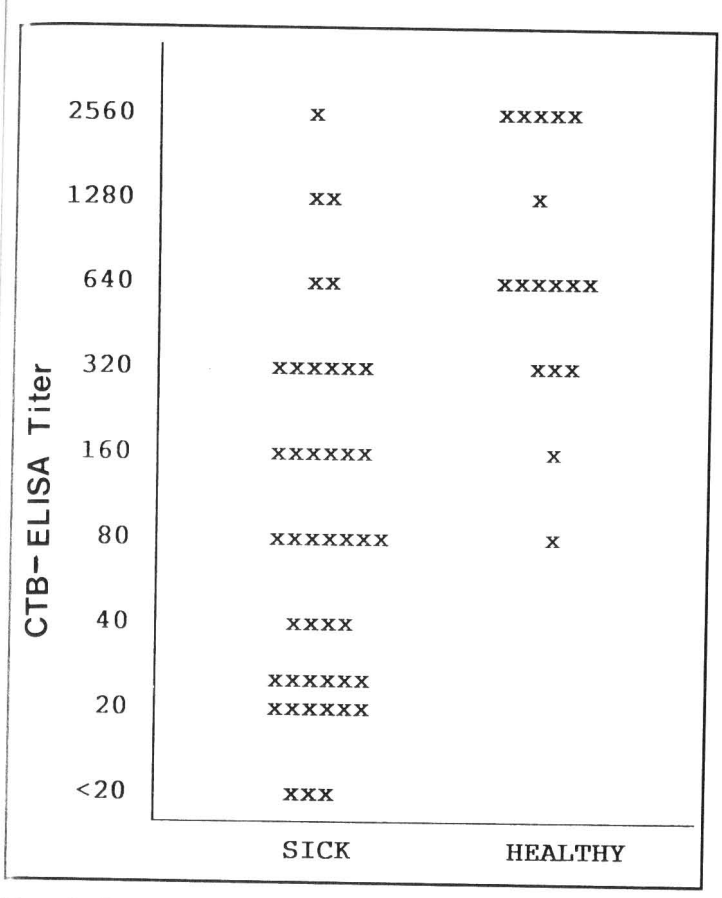


Figure 6—Results of the complex trapping/blocking ELISA. Titer of individual sick (n = 43) and healthy (n = 17) cats is shown.

Discussion

In addition to lymphadenopathy, transient hematologic abnormalities and a short period of fever, reproducible clinical signs of disease have not yet been observed after experimentally induced infection of cats with FIV.[4] Also the 2 cats of this study did not manifest clinical signs of infection for more than 2 years. However, persistent leukopenia, mainly attributable to lymphopenia, developed. In a previous study,[6] we reported impairment of immunologic functions in these 2 cats. The fact that they were housed under optimal hygienic conditions could be one of the reasons that the disease took so long to develop. Cats such as these may be important for studying the role of disease-potentiating cofactors in development of the acquired immunodeficiency syndrome-like stage of FIV infection. A more severe disease course has already been shown to develop in cats co-infected with FIV and FeLV.[15]

The protein composition of FIV resembles that of HIV. A major core protein of 24 kDa has been identified, as have minor core proteins of 15 and 10 kDa, along with their 50-kDa precursor protein. The major envelope protein is glycosylated and has Mr of 120 kDa as determined in infected lymphocyte cultures[16]; in addition, a transmembrane glycoprotein of 43 kDa is recognized.[10,16,17] In CRFK cells, a 100-kDa env protein and its 130-kDa precursor can be identified.[18,g] Detection of the immune response directed against individual FIV proteins depends on the assay used. In sequentially obtained samples from our experimentally inoculated cats, first antibody responses were directed against the env protein gp 120 and the gag protein p15; both activities could be detected by RIPA as early as PIW 2. Immunoblotting detected the earliest reaction (PIW 3 to 5) against the gag proteins (p24 and p15). Lack of reaction with the env protein by immunoblotting could be attributable to a quantitative phenomenon—loss of outer env proteins during virus purification. Using crude lysates of FIV-infected cells, a richer source of env protein, Hosie et al[10] were able to detect anti-env responses by immunoblotting. Probably, inefficient transfer of higher Mr proteins to the nitrocellulose filter, also is of importance; they remain in the gel, and were visualized after blotting by one of the authors (HFE) by staining the gel with Coomassie brilliant blue. In another study with FIV, HIV, and an ovine lentivirus, the immunoblotting technique was also shown to be insensitive for detecting antibodies against env proteins.[8,9]

The pattern of antibody responses during FIV infection as described here and by others[8] is comparable to that seen during HIV infection. Again, RIPA detected anti-env antibodies that remained occult by immunoblotting; anti-env and anticore antibodies were detected simultaneously.[10]

Two findings from the study of antibodies in field-infected cats should be emphasized—the quantitative differences in response against the core proteins, and the observation that some sera recognized only the env protein. It is unlikely that the titer differences determined by use of the p24-specific CTB-ELISA reflect strain differences. In the FIV strains analyzed so far, homologies of > 95% in the gag gene were found.[19] All sera recognizing p24 by immunoblotting competed with the binding of both MAB used in the CTB ELISA (data not shown), which therefore seem to recognize conserved epitopes on p24. Healthy cats have higher core antibody titer than do sick cats, an observation that could be of prognostic value. In people with HIV infection, antibodies to core proteins diminished before or at the onset of symptoms,[20] and their decrease correlated to disease progression.[21] Longitudinal studies of healthy FIV-infected cats are needed to establish whether the same is true for this infection. Also, the question of a relation between decreasing anticore antibodies and increasing p24 antigen in serum, as has been found in human patients with acquired immune deficiency syndrome, should be addressed.

Not all infected cats had antibodies against core antigen, whereas antibodies directed against env proteins were invariably detected by RIPA in IFA-positive field sera. Also O'Connor et al[8] found an anti-env response in all FIV-positive sera, and Hosie et al[10] mentioned anticore protein-positive sera that did not react to the env protein. However, these sera had been tested only by immunoblotting which would not detect discontinuous epitopes. In our study, 3 of 76 sera showed no reaction against core antigen, and reacted exclusively with the env protein as evidenced by RIPA; 2 of them also tested negative by CTB-ELISA. The new generation of diagnostic assays based on *gag* gene products only will be unable to detect a certain percentage of infected cats. Other proteins should be incorporated to make the assay more sensitive. We suggest the env protein, although it seems the least obvious one, because it is subjected to antigenic drift. However, the

[g] E. Verschoor. Personal communication.

consistent response found in all sera of this study justifies this postulate.

References

1. Pedersen NC, Ho E. Brown ML, et al. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* 1987;235:790–793.

2. Yamamoto JK, Hansen H, Ho EW, et al. Epidemiologic and clinical aspects of feline immunodeficiency virus infection in cats from the continental United States and Canada and possible mode of transmission. *J Am Vet Med Assoc* 1989;194:2–13–220.

3. Hopper CD, Sparkes AH, Gruffyd-Jones TJ, et al. Clinical and laboratory findings in cats infected with feline immunodeficiency virus in cats in the United Kingdom. *Vet Rec* 1989;125:293–297.

4. Yamamoto JK, Sparger E, Ho EW, et al. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *Am J Vet Res* 1988;49:1246–1258.

5. Ishida T, Tomoda I. Clinical staging of feline immunodeficiency virus infection. *Jpn J Vet Sci* 1990;52:645–648.

6. Siebelink KHJ, Chu I, Rimmelzwaan GF, et al. Feline immunodeficiency virus (FIV) infection in the cat as a model for HIV infection in man: FIV-induced impairment of immune function. *AIDS Res Hum Retrovir* 1990;6:1373–1378.

7. Barlough JE, Ackley CD, George JW, et al. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. *J AIDS* 1991;4:219–227.

8. O'Connor Jr TP, Tanguay S, Steinman R, et al. Development and evaluation of immunoassay for detection of antibodies to feline T-lymphotropic lentivirus (feline immunodeficiency virus). *J Clin Microbiol* 1989;27:474–479.

9. Saah AJ, Farzadegan H, Fox R, et al. Detection of early antibodies in human immunodeficiency virus infection by enzyme-linked immunosorbent assay, western blot and radioimmunoprecipitation. *J Clin Microbiol* 1987;25:1605–1610.

10. Hosie MJ, Jarrett O. Serological responses of cats to feline immunodeficiency virus. *AIDS* 1990;4:215–220.

11. Lange JMA, de Wolff F, Goudsmit J. Markers for progression in HIV infection. *AIDS* 1989;3(Suppl 1):S153–S160.

12. Egberink HF, Lutz H, Horzinek MC. Use of western blot and radioimmunoprecipitation for diagnosis of feline leukemia and feline immunodeficiency virus infection. *J Am Vet Med Assoc* 1991;199:1339–1342.

13. Houwers DJ, Schaake J. An improved ELISA for the detection of antibodies to ovine and caprine lentiviruses employing monoclonal antibodies in a one-step assay. *J Immunol Methods* 1987;98:151–154.

14. Conover WJ. Practical nonparametric statistics. New York: John Wiley & Sons, 1971;224–237.

15. Pedersen NC, Torten M, Rideout B, et al. Feline leukemia virus infection as a potentiating cofactor for the primary and secondary stages of experimentally induced feline immunodeficiency virus infection. *J Virol* 1990;64:598–606.

16. Egberink HF, Ederveen J, Montelaro RC, et al. Intracellular proteins of feline immunodeficiency virus (FIV) and their antigenic relationship to equine infectious anemia virus (EIAV). *J Gen Virol* 1989;71:739–743.

17. Steinman R, Dombrowski J, O'Connor T, et al. Biochemical and immunological characterization of the major structural proteins of feline immunodeficiency virus. *J Gen Virol* 1989;71:701–706.

18. Olmsted RA, Hirsch VM, Purcell RH, et al. Nucleotide sequence analysis of feline immunodeficiency virus: genome organization and relationship to other lentiviruses. *Proc Natl Acad Sci USA* 1989;86:8088–8092.

19. Phillips TR, Talbott RL, Lamont C, et al. Comparison of two host cell range variants of feline immunodeficiency virus. *J Virol* 1990;64:4605–4613.

20. Lange JMA, Coutinho RA, Krone WJA, et al. Distinct IgG recognition patterns during progression of subclinical and clinical infection with lymphadenopathy-associated virus/human T-lymphotropic virus. *Br Med J* 1986;292:228–230.

21. Weber JN, Clapham PR, Weiss RA. Human immunodeficiency virus infection in two cohorts of homosexual men: neutralizing sera and association of anti-gag antibody with prognosis. *Lancet* 1987;1:119–122.