In the labeled antigen configuration, purified viral antigens are coated on the solid phase and used to prepare the enzyme-labeled antigen conjugate (Fig 6). Because antibodies have 2 identical binding sites, specific antibody in the test sample can bind simultaneously to the antigen coated on the solid phase and to the enzyme-labeled antigen. After a wash step, bound immune complexes are detected using the substrate/chromogen reagent. A membrane-based ELISA, using a labeled antigen configuration for FIV antibody and a direct antigen configuration for FeLV p27 has been developed. It incorporates an FeLV antigen ELISA, an FIV antibody ELISA, and negative and positive controls. The combination assay allows simultaneous testing for both diseases, using the same sample. This combination test has wide use for diagnosis of FeLV and FIV infections, and is particularly suited to testing sick cats or those judged to be at risk for such retroviral infections.

Immunoassays have proved to be useful diagnostic tools for many infectious diseases. The ELISA is an extremely versatile type of immunoassay that can be configured in a number of ways and delivered in several formats. These formats allow for batch or individual testing and have been shown to be particularly useful for diagnosing feline retroviral infection.

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Use of western blot and radioimmunoprecipitation for diagnosis of feline leukemia and feline immunodeficiency virus infections

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Summary: *The uses and limitations of the western blot (WB) and radioimmunoprecipitation assay (RIPA) techniques for study of feline immunodeficiency virus (FIV) and FeLV were evaluated. Western blot analysis was used to detect antigenic relatedness between the 2 lentiviruses. Using a rabbit serum directed against p26 of the equine infectious anemia virus (EIAV) and anti-EIAV horse serum obtained from an infected horse, cross-reactivity with p24 of FIV was revealed. Cat sera obtained late after experimentally induced FIV infection recognized p26 of EIAV, which indicates reciprocal cross-reactivity. For RIPA, FIV was metabolically labeled, and virus-coded proteins were identified, using immunoprecipitation. Polypeptides with apparent molecular mass of about 15, 24, 43, 50, 120, and 160 kilodaltons were detected. An additional polypeptide of 10 kilodaltons was found only by use of WB analysis.*

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Well studied lentiviruses (members of the Retroviridae family) in animals are equine infectious anemia virus (EIAV), Visna virus (VV) and caprine arthritis-encephalitis virus (CAEV); sequence homologies in the *gag* and *pol* genes between these viruses and the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) have been reported.[1] In addition, nonreciprocal antigenic cross-

reactivity was observed between the *gag* gene products p24 of HIV and p26 of EIAV. Cross-reactivity was not detected between VV or CAEV and EIAV or HIV, respectively.[2] Also, antigenic relationships between feline immunodeficiency virus (FIV) and HIV-1, HIV-2, simian immunodeficiency virus (SIV), CAEV, and VV have not been detected when antibodies from naturally infected hosts were used.[3] In a later study, however, rabbit antisera to CAEV and VV were found to react with the putative core protein of FIV.[4] Recently, we documented reciprocal heterologous recognition of the major core proteins between FIV and EIAV.[5] We evaluated the uses and limitations of western blot (WB) and radioimmunoprecipitation (RIPA).

The FIV was propagated in thymocytes or blood lymphocytes from specific-pathogen-free (SPF) cats; cells were stimulated with concanavalin A (conA; 5 μg/ml) and recombinant human interleukin 2 (IL-2; 100 U/ml), as described by Pedersen et al.[6] Our search for an antigenic relationship of the FIV-specific proteins with those of established lentiviruses focused on EIAV, which had not been included in an earlier comparative study.[3] When using a rabbit immune serum directed against the p26 of EIAV,[7] weak reaction was noticed in immunofluorescence assays on FIV-infected Crandell feline kidney (CRFK) cells. Therefore, WB was performed to examine the reaction of homologous and heterologous sera with proteins of gradient-purified EIAV and FIV; the proper control preparations were included (EIAV-negative horse serum and FIV-negative cat serum). Electrophoresis and transfer of the proteins to nitrocellulose filters was performed in routine manner as follows.

The viral proteins are separated by electrophoresis in 12.5% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE), then electrophoretically transferred to nitrocellulose sheets. After transfer, the nitrocellulose filters are blocked for 2 hours at 20 to 22 C (room temperature), using gelatin buffer. The filters are then incubated at 37 C on a rocker platform, with the antisera diluted in gelatin buffer. After several washings the filters are incubated with horseradish peroxidase (HRP)-conjugated anti-species IgG. They are then washed again, after which, the antigen-antibody reaction is visualized, using 4-chloro-1-naphthol as a chromogen. The enzyme-substrate reaction is stopped by submerging the filters in 3% trichloroacetic acid.[8]

As described,[5] the anti-FIV cat serum recognized the 24 and 15-kilodalton (kDa) proteins and an additional minor protein of about 10 kDa. The latter protein, presumably another cleavage product of the gag precursor, was not detectable in L-[^{35}S]methionine-labeled cell lysates of FIV-infected lymphocytes. The high molecular weight proteins were not visualized, probably because of loss of the knobs from the virion membrane during purification and poor transfer to the nitrocellulose filter.[9]

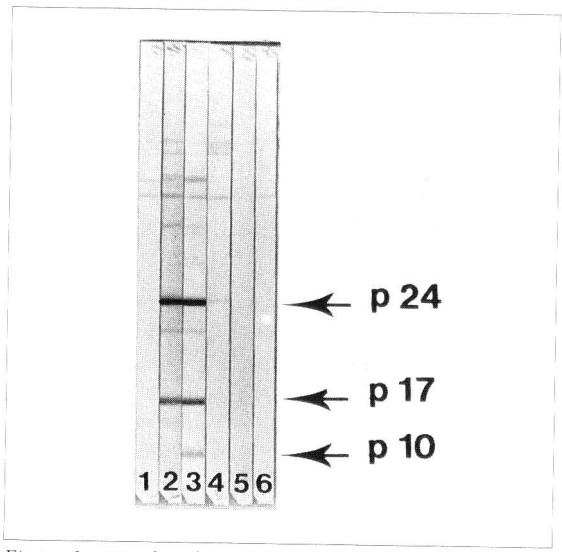

Figure 1—Results of western blot analysis performed using different cat sera: gradient-purified feline immunodeficiency virus (FIV; 1 μg/lane) was analyzed by use of sodium dodecyl sulphate polyacrylamide gel electrophoresis. Lane 1 = conjugate control; lane 2 = serum obtained from a cat 4 weeks after experimental induction of FIV infection; lane 3 = serum from the same cat, 6 weeks after infection; lanes 4, 5, and 6 = serum from a cat with pre-existing FeLV infection 3 (lane 4), 4 (lane 5), and 5 (lane 6) weeks after experimentally induced FIV infection. Antibodies were not detected in this cat, from which FIV could be isolated 3 times at weekly intervals.

The EIAV-specific proteins were recognized by homologous horse serum. The rabbit anti-p26 EIAV and the cat anti-FIV serum recognized the p26 of EIAV. Also, the rabbit and horse anti-EIAV sera recognized the p24 of FIV, which indicates two-way cross-reactivity. When sera from serially collected blood samples of experimentally FIV-infected cats were tested, it appeared that p26 of EIAV was recognized only after postinfection (PI) week 14, whereas the homologous reaction was visible from PI week 6 onward. These observations indicate that the epitopes conserved between FIV and EIAV may not be immunodominant, and prolonged exposure to the immune system is required for antibodies to develop.

On the basis of serologic cross-reactivity at the gag protein level, it was proposed to distinguish 2 groups within the lentiviruses, the first one including VV, CAEV and pleuropneumonia virus of sheep, the second group comprising HIV, SIV, and EIAV.[2] Our results indicate that FIV should be included in the second group. Also one of us (MCH) found that the major core antigen of the bovine immunodeficiency virus (BIV; 10) is recognized by rabbit anti-EIAV-p26 serum (data not shown). The aforementioned FIV-EIAV relationship has been established independently in another laboratory.[11]

In view of the widespread development of antibodies against FeLV and feline syncytial forming virus (FeSFV) in the random cat population, sera from cats experimentally infected with these viruses were tested for possible cross-reactions with

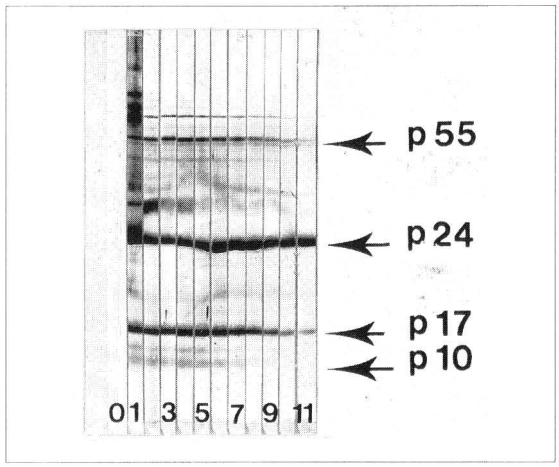

Figure 2—Results of western blot analysis performed using serial serum dilutions. Lane 0 = negative control—serum from a specific-pathogen-free cat; lane 1 = positive control—serum obtained from a cat with experimentally induced FIV infection; lanes 2 to 11 = serial twofold dilutions (1:20 [lane 2] to 1:10,240 [lane 11]) of a serum from a cat with natural FIV infection. Reactivity with: the p10 band of FIV disappears at serum dilution 1:640; the p17 band diminishes at the highest dilution; and the p24 band remains the same throughout the series.

FIV by use of WB. As reported previously,[12] arrays of bands were detected with little resolution in the high molecular weight range. However, proteins of 42, 24, and 15 kDa were recognized by anti-FIV serum. Sera obtained from cats after experimentally induced infection with FeSFV had positive results in the homologous immunofluorescence test and recognized many high molecular weight proteins, as well as a 42-kDa polypeptide. The nature of this protein was not examined; it may be of viral origin, having epitopes common to FIV and FeSFV (eg, on the transmembrane domain of the *env* gene product that was shown to be highly conserved.[13] Alternatively, it may represent a host cell protein. One candidate would be actin (MW, 42,000), which has been shown to contaminate FeLV preparations even after gradient purification and which may induce antibodies in cats after natural infection with FeLV.[14]

It is obvious that the assay may fail to detect antibodies in immunosuppressed cats as a consequence of a concurrent FeLV infection (Fig 1; lanes 4-6). Also, FIV may be isolated in culture (early after infection), while WB results are still negative. Some authors[15] report as many as 15% of virus-positive, antibody-negative cats in the field (as tested by ELISA). An estimate of the relative antibody titer against individual virion proteins can be made by use of WB (Fig 2).

An explanation of the RIPA procedure is as follows. Radioactive label is added to FIV-infected thymocyte and lymphocyte cultures at 5 and 8 days, respectively, when reverse transcriptase (RT) activity is high. About 2×10^6 viable cells are pelleted, washed once in phosphate-buffered saline solution, and resuspended in methionine-deficient minimal essential medium (MEM) supplemented with fetal bovine serum; then L-[^{35}S] methionine is added. Cells are incubated overnight, pelleted, and lysed. For glycoprotein labeling, cells are incubated in MEM containing [^{3}H]glucosamine hydrochloride.

Lysates of labeled infected cells are incubated with sera from cats before and after experimental induction of FIV infection. Preparations containing sufficient virus-specific radioactivity are diluted with a lysis buffer and mixed with the immune serum. Immune complexes are allowed to form overnight at 4 C, then precipitated, using formaldehyde-fixed *Staphylococcus aureus* cells. Precipitates are washed and resuspended in a sample buffer, and proteins are analyzed by use of SDS-PAGE.[16]

We have documented that sera from FIV-infected cats recognize proteins with apparent molecular mass of 15, 24, 43, 50, 120 and 160 kDa in lymphocyte lysates. In contrast to p120, the 160-kDa polypeptide was not always detectable. The 120-kDa protein can be labeled with [^{3}H]glucosamine. When FIV-infected lymphocytes were grown in the presence of tunicamycin, an inhibitor of N-linked glycosylation, gp120 was lacking and a new polypeptide of about 75 kDa appeared in the gel; these data indicate that p75 may be the unglycosylated precursor of gp120.[5]

The 15- and 24-kDa proteins probably represent core proteins with their 50-kDa precursor (*gag* gene products); evidence for this assumption came from so-called pulse-chase experiments. Cells were pulse-labeled with L-[^{35}S]methionine for 0.5 hour, and incubated further with twice the amount of nonradioactive methionine for variable periods (chase). When comparing the results from the 0.5- to 4-hour chase periods, the intensity of the prominent 50-kDa band was seen to decrease, whereas the 24- and 15-kDa bands increased.[5]

In HIV, a highly glycosylated precursor protein (gp160) is processed to yield an outer membrane protein of 120 kDa and a transmembrane protein of 41 kDa.[17] In our pulse-chase experiment, the 160-kDa band was only faintly visible during the first 0.5 hour of the chase period, and specific processing products were not detected. The decrease in intensity of gp120 during the chase may be attributable to release of the protein from the cells.

The 43-kDa species is considered to be the matrix protein (*env* gene product). In contrast to the gp41 protein of HIV and the p43 of FIV in CRFK cells,[11] the 43-kDa protein of FIV in lymphocytes could not be labeled with [^{3}H]glucosamine.

As indicated by the 2 examples, RIPA and WB are powerful techniques that complement the arsenal of serologic methods used in virology. Like any other technique of this kind, they can be used for characterization of either component in the immune complex: the antigen (eg, identification of virus-coded proteins; assessment of antigenic rela-

tionships between viruses; the fate of a translation product in an infected cell) or the antibody (response against individual proteins of a virus during infection, as a function of disease progression; confirmation of ELISA or immunofluorescence results; specific assessment of monoclonal antibodies).

The assays differ in one important aspect: whereas native proteins released from the virion by mild detergents are allowed to react with the antibody before electrophoresis in RIPA, SDS-denatured polypeptides are transferred to the nitrocellulose filter in the WB procedure. Thus, conformational and discontinuous epitopes may be recognized by use of the former and escape detection by use of the latter technique. This is not necessarily a disadvantage of WB because comparative assays may provide information about the nature of an epitope, especially when monoclonal antibodies are used.

Purification of FIV results in large losses of gp120, as has also been reported for HIV.[9] However, using WB on lysates of FIV-infected cells, Hosie and Jarrett[18] obtained satisfactory signals in the high molecular weight region. In horizontal studies, we found that cats mount an antibody response first against the gp120, then against the *gag* gene products; the latter are easily detected by use of WB, but RIPA gives cleaner pictures for gp120. Some cats have faint responses or none against the major core protein. On the other hand, an anti-env response is invariably detectably by RIPA. Analogous to AIDS in human being, a decrease in anti-core antibodies was noticed in one cat progressing toward disease manifestation.

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