Immuno-chromatographic assay for diagnosis of feline leukemia virus infection

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Abstract

Feline leukemia virus (FeLV) infectious disease is one of feline infection diseases spreading broadly all over the world. For bedside diagnosis of FeLV infectious disease, an immuno-chromatographic assay was investigated. Five different monoclonal antibodies were developed against the major core protein FeLV-p27. Among them, the combination of FL6 and FL12, which had little epitopic overlap each other, showed the highest sensitivity with no cross-reaction to the other feline virus antigens when they were employed to the immuno-chromatographic assay. The system had a practical detection limit of 0.5 ng of FeLV-p27 per 0.1 ml of feline sera within 15 min. In comparison with clinical standard methods, the system gave rapidly and accurately the same diagnosis with neither false negative nor false positive. Moreover, it did not need any pretreatment of blood specimen.

Introduction

Feline leukemia virus (FeLV) was discovered from cats that lived in a cluster-household where several cats had developed lymphosarcoma (Jarret et al. 1964). The virus was first isolated from the serum of cat with lymphosarcoma by Kawakami et al. (1967) and was characterized as an exogenous retrovirus, 115 nm in diameter, containing a protein core with single-stranded RNA protected by a lipoprotein envelope. Based on similarities in nucleotide sequences, it has been determined that the evolutionary source of the virus was from an ancestor of the rat (Benveniste et al. 1975). Although the period of disease development is highly variable, 83% of FeLV infected cats die within 3 years (McClelland et al. 1980). FeLV is now considered to be the most prevalent cause of severe

illness and death in domestic cats. The incidence of FeLV infection in cats in a large urban area was 40%, contrasting with 6% in the surrounding rural area. This indicates that FeLV infection is common and is horizontally transmitted (Rogerson et al. 1975). Non-infected cats living in close proximity to cats shedding FeLV have a greatly increased risk of infection, and once infected, the cat has an approximately 900 times greater risk of developing lymphosarcoma. It should be possible to break the cycle of horizontal transmission by vaccination or by selectively segregating or eliminating FeLV positive cats from the cat population. For this purpose, simple and rapid diagnosis is important. FeLV has been detected or measured quantitatively by passive hemagglutination test (Sibal et al. 1970), complement fixation test (Sarma et al. 1971), mixed hemadsorption test (Maruyama

et al. 1971), immunofluorescent assay (IFA) (Hardy et al. 1973), focus formation assay using cloned cat cell line C81 (CC81) (Fischinger et al. 1974), mixed immunoglobulin rosette technique (MIA) (Mackey et al. 1975), ELISA using polyclonal antibody (Lutz et al. 1980a), and ELISA using monoclonal antibody (Lutz et al. 1983a, 1983b). All of these methods require either multiple-step procedures or a long assay time. Immunochromatographic assay is a version of immunoassay that can be used for point-of-care examination of patients (Chen et al. 1987; Lou et al. 1993) and also at field test of, for instance, pregnancy in cattle (Laitinen and Vuento 1996). Here, we report on a rapid, one-step, non-instrumented, immunochromatographic assay method that measures FeLV-p27, a component of the shell of the viral core, for feline leukemia diagnosis.

Materials and methods

Viruses and host cells

Four viruses known to infect cats were used in this study. FeLV3281 cells (Donahue et al. 1988; Grant and Michalek 1981), a lymphoblastoid cell line, are a FeLV subgroup A (Sarma and Log 1973) carrier. The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine, 14 mM NaHCO₃, and antibiotics (100,000 units/l of penicillin and 0.1 g/l of streptomycin). Feline immunodeficiency virus (FIV) (Yamamoto et al. 1988), feline panleukopenia virus (FPLV) (Johnson et al. 1974), and feline infectious peritonitis virus (FIPV) (Boyle et al. 1984) were grown in Crandell-Reese feline kidney (CRFK) cells (Crandell et al. 1973) in minimal essential medium supplemented with 5% fetal bovine serum, 10% toripose phosphate brose, 4 mM glutamine, 14 mM NaHCO₃ and antibiotics.

Preparation and purification of virus

For the purification of FeLV particles, cultured medium harvested from FeLV3281 cells culture was centrifuged at 400 g for 10 min to remove cell debris. The clarified specimen was precipitated into pellet by centrifugation at 66,000 g for

30 min. The pellet was resuspended in phosphate buffered saline (PBS) and was finally purified in a 25–50% sucrose density gradient in a SW50.1 rotor (Beckman Coulter, Fullerton, USA) at 25,000 rpm for 2 h at 4 °C (Kawakami et al. 1967).

For the purification of FeLV-p27, the method for core protein purification of type C RNA tumor viruses by Strand and August (1976) was employed. Purified FeLV particles were disrupted by incubation at 4 °C for 30 min with 0.05 M Tris–HCl buffer (pH 8.9) containing 0.01 M EDTA, 1.0 M NaCl, and 0.5% Triton X-100, and then centrifuged at 67,000 g for 30 min. The supernatant was dialyzed against 100 vol. of 0.01 M Tris–HCl buffer (pH 8.9) containing 0.1 M KCl, and then applied to a phosphate cellulose column. Bound materials were eluted with the Tris–HCl buffer containing KCl gradient. The concentration of KCl ranged from 0.1 to 1 M. The FeLV-p27 containing fraction was specified by western analysis as previously described (Eto et al. 1991a). In brief, each fraction was applied to SDS–PAGE and the protein bands on the gel were transferred to nitrocellulose filter (Schleicher and Schuell, Dassel, Germany). The band was visualized by using goat polyclonal antibody for FeLV-p27 (Fitzgerald Industries International Inc., Concord, USA). FeLV-p27 was eluted with Tris–HCl buffer including 565 mM KCl, and dialyzed against 100 vol. of PBS.

Immunization and hybridization

BALB/c mice were immunized with 30 µg FeLV-p27 given intraperitoneally in complete Freund's adjuvant. Three booster injections containing 30 µg FeLV-p27 in incomplete Freund's adjuvant were given intraperitoneally every 10 days. On the third day after the final immunization, splenocytes were isolated and fused with a HAT (hypoxanthine, aminopterin, thymidine) sensitive mouse myeloma cell line, P3-X63-Ag8-U1 cells, by the PEG method (Galfrè and Milstein 1981). Hybridomas producing MAb reactive to FeLV-p27 were cloned from twice to five times by the limited dilution method (Goding 1980). Established hybridomas were cultured in the serum free DF medium (Barnes and Sato 1980) supplemented with 15 mM HEPES, 14 mM NaHCO₃, and

antibiotics same as above for RPMI medium, 10 $\mu\text{g/ml}$ insulin, 35 $\mu\text{g/ml}$ of transferrin, 20 μM ethanolamine and 25 nM selenium (ITES)(Murakami et al. 1982). Monoclonal antibodies (MAbs) were purified by using a Hi-trap Protein G column (Amersham Biosciences, Piscataway, USA), and FL6 was biotinylated by using a Biotinylation kit (Amersham Biosciences).

Reactivity of MAbs to FeLV-p27 or virus particles

The reactivity of MAbs to FeLV-p27 or virus particles was determined by an ELISA as previously described (Eto et al. 1991b). In brief, a 96 well-immunoplate which had been adsorbed by 100 μl of FeLV-p27 or virus particles was treated with 200 μl of four times diluted solution of Block Ace (Dainippon pharmaceutical Co. Ltd., Osaka, Japan) for 2 h. The plate was washed with PBS containing 0.05% Tween 20 (TPBS) and reacted with 50 μl of testing MAb for 1 h. The plate was washed by TPBS and then bound MAb was reacted with 100 μl of 2000-times-diluted peroxidase-labelled anti-mouse IgG (Organon Teknika N.V. Cappel Products, Westchester, USA) for 1 h. After washing the plate with TPBS, 100 μl of substrate solution [0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg/ml ABTS] was added to each well and incubated for 30 min. Absorbance at 405 and 490 nm was measured with a EAR400 electrophotometer (SLT-LABINSTRUMENTS, Salzburg, Austria). All the above reactions were carried out at 37 °C.

Competition assay

A competing examination was used to choose a set of the antibodies suitable for a sandwich assay. The dosage of the biotin-labelled FL6 which reacted to 50% of FeLV-p27 (300 ng/0.1 ml) was 250 ng/25 μl . MAb of 10^n ($n = -1 \sim 2$)-fold concentration of biotinylated FL6 was mixed with the 250 ng of labelled FL6. Then the mixture was added to the immunoplate which was coated with FeLV-p27. After 1 h, the bound biotinylated FL6 was assayed with peroxidase-labelled streptavidin. The percentage of inhibition of biotinylated FL6

binding to FeLV-p27 was calculated according to the following formula:

$$\% \text{inhibition} = [1 - (\text{OD}_E - \text{OD}_N) / (\text{OD}_P - \text{OD}_N)] \times 100,$$

where OD_P (OD in positive control) was obtained from mixture of labelled FL6 (25 μl) and PBS (25 μl) instead of non-labelled MAb. OD_E means OD in experiment, OD_N means OD in negative control.

Construction of membrane strip

The purified MAb was conjugated with colloidal gold for colorimetric label (Paek et al. 1999). The MAb solution dialyzed against 10 mM sodium borate buffer, pH 9.0, was diluted to 100 $\mu\text{g/ml}$ with distilled water. Five milliliters of the solution was added dropwisely to 100 ml of a gold solution (British BioCell International) while stirring rapidly. After 30 min the residual surfaces of the gold particles were blocked by adding 10% BSA for 30 min. The gold particles were precipitated by centrifugation at 8000 g for 45 min and the supernatant was then removed. Collected MAb-gold conjugates were suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 10% BSA, 10% lactose and 0.1% sodium azide, and it was diluted so that OD530 value became 6.0. The conjugated MAb was made to soak into gold colloid absorption pat (Millipore, Billerica, USA) uniformly, and was freeze-dried. The chromatographic strip was built as follows: An anti-FeLV MAb and anti-mouse IgG antibody were coated in filamentous of 1 mm width in nitrocellulose membrane (Millipore) for chromatograph. The membrane was blocked with 1% casein, rinsed with water, protected with 10% glycerol, and then air dried. The membrane and pads were arranged from the bottom as shown in Figure 1: cellulose membrane (ADVANTEC, Dublin, USA) for sample application pad, the conjugated antibody adsorption pad, the nitrocellulose membrane with immobilized antibodies on the surface, and cellulose membrane (ADVANTEC) as absorbent pad. The membranes in the order were partially superimposed and attached onto a vinyl chloride sheet (Wako Pure Chemical Industries, Osaka, Japan) using a both-sided tape.

Figure 1. The membrane strip assay system based on immuno-chromatography. The system consists of four parts: (1) cellulose membrane for sample application pad; (2) grass fiber membrane for gold labelled antibody absorption pad; (3) nitrocellulose membrane with immobilized antibodies against FeLV-p27 or mouse IgG; and (4) cellulose membrane for absorbent pad. Some blood containing virus protein is introduced into the strip by the capillary action and the binding between the FeLV-p27 and the labelled anti-FeLV-p27 MAb takes place. This binding complex is transferred by the capillary flow to the nitrocellulose membrane. Then the complex reacts with the immobilized anti-FeLV-p27 MAb for result signal generation. Moreover, the complex reacts with the immobilized anti-mouse IgG antibody for reference signal generation.

Lower limit of detection

The membrane strips were assembled by three labelled MABs in combination with four catching MABs. FeLV-p27 antigen purified from the virus secreted by FeLV3281 cells was diluted with serum which did not include feline antigen. One hundred μ l of serum including antigen was added onto sample application pad of the membrane strip, and reacted for 15 min at room temperature. When gold labelled anti-FeLV-p27 MAB was captured with anti-mouse IgG antibody on the reference signal line, gold was accumulated on the line and visualized. In similar, the complex of gold labelled anti-FeLV-p27 MAB and the antigen reacted with immobilized anti-FeLV-p27 MAB on the result signal line, the judgment was done by visual observation of signal generation.

Clinical standard methods for diagnosis of FeLV infection

Streak specimen of peripheral bloods were provided by Drs. Kiyotaka and Eiko Kushima at

Seiho animal hospital. Detection of FeLV group specific antigen (gsa) was done by IFA method (Hardy et al. 1973). Anti-FeLV dog antisera (COSMO BIO, Tokyo, Japan) were used for the primary antibody, and anti-dog IgG labelled with fluorescein isothiocyanate was used for the second antibody. FeLV isolation tests were done by CRFK cells. After inoculation at 1×10^5 cells/ml and cultivation for 24 h, the cells were treated with DEAE-Dextran (50 μ g/ml) for 1 h, and infected for 2 h with a specimen which was diluted into 10 times in culture medium. After removing the specimen, cells were cultured for 6 days and FeLV gsa was detected by IFA method. Quantitative assay for FeLV was done by focus formation assay (Tsujimoto et al. 1981) by using CC81 cells (Fischinger et al. 1974). The virus titer was expressed as focus inducing units (FIU).

Results

Characterization of MABs to FeLV-p27

After two fusion experiments, five hybridomas producing MAB reactive to FeLV-p27 were isolated. All MABs (FL6, FL12, FL18, FL25, and FL30) are IgG1 with κ light chains. Figure 2 shows the reactivities of MABs to FeLV-p27. There was a little difference among these five MABs. FL12 had reactivity about twice as active as FL6, FL18, and FL25. All MABs developed here could detect 1 μ g/ml of FeLV-p27 at 250 ng, indicating they have sufficient affinity with FeLV-

Figure 2. Reactivities of MABs against FeLV-p27. FL6 (●), FL12 (▲), FL18 (□), FL25 (▽), and FL30 (△), 250 ng of each was added to wells of 96-well immunoplate coated with various concentrations of purified FeLV-p27.

Figure 3. Reactivities of MAbs against various viruses causing feline diseases. Each MAb (750 ng) was added to wells of 96-well immunoplate coated with purified virus particles of FIV (a), FPLV (b), FeLV (c), and FIPV (d). For the symbols, see Figure 2.

p27. Figure 3 shows the reactivities of MAbs against various viral particles. These MAbs were able to detect 30 $\mu\text{g/ml}$ of FeLV particles at 750 ng, and shows no cross-reactivity with FIV, FPLV, and FIPV that caused other feline diseases. From the high detectability without cross-reaction, it was thought that these MAbs could be used as a diagnostic agent. Sandwich assay for antigen detection needs a set of two MAbs, which recognize different epitopes. In order to choose the set, we applied competitive assay by using at first FL6 as a labelled antibody. Competitors were conveniently divided into three groups from the result shown in Figure 4. FL30 competitively inhibited the binding of biotinylated FL6 to FeLV-p27, indicating to recognize the same or close epitope, which FL6 recognizes. Because the reaction of the biotinylated antibody was not inhibited with FL18 and FL25, the epitopes recognizable with these MAbs may differ from the one recognized with FL6. It is suggested that the epitope of FL12 is near to the epitope of FL6 since FL12 had the intermediate reactivity in comparison with the former two groups. Thus the FeLV-p27 may be validly measured by using the MAb set of FL6 and FL18, or combination of FL6 and FL25.

Figure 4. Competition of biotinylated FL6 binding to FeLV-p27 by various MAbs. Biotinylated FL6 (250 ng) was mixed with each MAb of dosage shown in graph. Then the mixture was added to 96-well immunoplate which was coated with 3 $\mu\text{g/ml}$ of FeLV-p27. For the symbols, see Figure 2.

FeLV-p27 detection limit and specificity of the membrane strip based on immuno-chromatography

Membrane strips based on immuno-chromatographic assay were constructed by using MAbs sets described above and then checked up on their detection limit and specificity. FL6, FL18 and FL25 were used for a gold colloid labelled antibody. In the case of FL12, it was inactivated with labelling. For a catching antibody, FL6, FL12, FL18 and FL25 were used. As shown in Table 1, both the combinations of FL6-FL18 and FL6-FL25 were able to detect FeLV-p27. The former combination had higher sensitivity than the other one. In addition, when FL6 was used as a gold colloid labelled antibody than FL18, detectability increased twofold. The combination of FL18 and FL25 was not able to detect the antigen, because the same epitope was recognized as expected from

Table 1. Detection limit of FeLV-p27 by immuno-chromatographic assay

	Catching antibody	FL6	FL12	FL18	FL25
Gold colloid labelled-antibody	FL6	—	0.5	5.0	15
	FL18	10	15	—	ND
	FL25	15	ND	ND	—

Data are shown by FeLV-p27 concentration (ng/100 μl); ND = not detected.

the result of Figure 4. When FL6 and FL12 were used as a gold colloid labelled antibody and a catching antibody, respectively, the highest sensibility was obtained, being detectable at 0.5 ng/0.1 ml of FeLV-p27. On the other hand, the specificity of the immuno-chromatographic membrane strip was examined by using blood or sera obtained from 15 cats that were infected with either FIV, FIPV or FPLV. As a result, the membrane strip did cross reaction with neither of these specimens (data not shown).

Comparison of methods for diagnosis of FeLV infection between the membrane strip and the other clinical standard methods

Comparison with the immuno-chromatographic membrane strip method and clinical standard approaches was done. For clinical standard approaches, three following methods were used: (1) IFA method detecting FeLV gsa on smear preparation of a peripheral blood; (2) isolation method of FeLV with CRFK cells, followed by detecting FeLV gsa, and (3) FIU measurement with CC81 cells. Seven cats were tested at Seiho animal hospital as shown in Table 2. Cat 1 and cat 2 were kept in the same house. They appeared to have caught a disease which may be unrelated to the FeLV infection, but were diagnosed as a subclinical infection with FeLV. Disease of these two cats was caused by opportunistic infections owing to depress immune mechanisms severely with FeLV infection. The third cat of anemia was diagnosed as a leukopenia by the FeLV infection. Cats from the fourth to the seventh were kept in the same

house. Although all the cats except for the seventh were healthy outwardly, they were inspected because the seventh cat had been diagnosed as a lymphosarcoma by the FeLV infection. As a result of examinations by clinical standard approaches of these specimens, FeLV was confirmed positive for all cats except the fourth. The fifth and the sixth cats were diagnosed as a subclinical infection of FeLV. Completely the same results were given when these specimens were inspected with the membrane strip. There were not the false positive or the false negative at all.

Discussion

To establish a simple and rapid diagnosis system for FeLV infection, we cloned five hybridomas producing MAbs reactive to FeLV-p27 and characterized them on reactivity to the virus. Because FIV, FPLV, and FIPV present clinical manifestations analogous to FeLV infections, clear differentiation is needed (Pedersen et al. 1977; Cotter et al. 1973). All the obtained MAbs reacted specifically with FeLV without cross-reactivity against those three viruses which are not immunogen. However, the reactivities of the MAbs to FeLV particles were lower than against FeLV-p27 (Figures 2 and 3). This is because p27 is a component of the shell of the viral core (Bolognesi et al. 1978). In general, core protein is not regularly expressed in the intact virion because it is masked by the presence of envelope. However, in the case of FeLV infection, the internal structural proteins are produced in great excess in the cytoplasm of the cell during the replication cycle (Hardy et al. 1969). Most of internal structural

Table 2. Comparison of the results of clinical standard methods and immuno-chromatographic assay

Case No	Diagnosis	Isolation of FeLV		Detection of FeLV gsa*	Detection of FeLV-p27 with immuno-chromatographic membrane strip
		In CRFK	FIU**/ml in CC81(log10)	IFA	
1	Subclinical infection	+	2.5	+	+
2	Subclinical infection	+	2.0	+	+
3	Leukopenia	+	1.5	+	+
4	Healthy	—	ND	—	—
5	Subclinical infection	+	1.7	+	+
6	Subclinical infection	+	5.0	+	+
7	LSA*** (Thymic)	+	5.5	+	+

gsa*, group specific antigen; FIU**, Focus inducing units; LSA***, Lymphosarcoma; +, Positive; —, Negative; ND, not detected.

proteins are never packaged into FeLV particles and remain inside the cell or become solubilized in plasma. For this reason, the major core protein p27 is present in particularly high amounts in the blood and is a good marker for the detection of FeLV infection (Lutz et al. 1980b).

Since the epitope for FL6 may be different from epitopes for FL18 and FL25, it seemed to be ideal combinations for a sandwich assay. In fact, these two combinations have sensitivity (5.0 ng/0.1 ml) more than required as described later. However, it is a combination of FL6 and FL12 that the highest sensitivity (0.5 ng/0.1 ml) was obtained. Perhaps, certain parameter except steric hindrance affected this greatly. The antibody that has high affinity is advantageous to catch antigen-antibody complex that consists of conjugated FL6 and FeLV-p27 when there is little steric hindrance. Since FL12 has higher affinity for the antigen than any other antibodies, may have made up for disadvantage about competitive inhibition produced by coexistence with FL6. Moreover, maximum binding of FL12 for the certain amount of antigen is larger than those of FL18 and FL25 (Figure 2). It seems that this thing acts profitably to generate strong signal. In this study, the binding affinity between antigen and antibody was measured by ELISA under equilibrium conditions. Because an immuno-chromatographic assay is performed under non-equilibrium condition, rate of binding becomes more important than dissociation constant. The better choice of MAbs will be done by measurements of kinetic rate constant for the binding with the use of surface plasmon resonance biosensor (SPR).

Since the mean value of FeLV-p27 included within infected feline serum is 480 ng/0.1 ml (60–1500 ng/0.1 ml) in a cat suffered from persistent viremia and 360 ng/0.1 ml (30–2800 ng/0.1 ml) in a transient viremia cat (Lutz et al. 1983b), the immuno-chromatographic strip which showed the detection limit of 0.5 ng of FeLV-p27 per 0.1 ml of feline sera is very useful for the practical diagnosis. As shown in the case of the fifth and the sixth cats in the Table 2, the membrane strip assay enabled us to detect FeLV even in outwardly healthy cat which has only a little virus. Because FeLV-p27 appears before IFA positive in the early period of viremia (Lutz et al. 1983b), it is important to disclose infected cat at such an early stage when infection to others is prevented. In addition, the strip has a practically high specificity; it does not

react to the other pathogenic viruses which show similar manifestation with FeLV infection.

In a field examination, the membrane strip assay was able to give equal diagnosis with three clinical standard methods. There were not the false positive or the false negative at all. This thing expresses reliability of the membrane strip assay, however, it will need further controlled trials. Clinical standard approaches need laboratory facilities and an inspection period for 5–7 days, but, in the membrane strip assay, it is possible to give a diagnosis at bedside within 15 min. It is well-known that this simplicity and speed are important in contagious sickness prevention. The membrane strip developed in this study has been employed for practical use by veterinarians in Japan.

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