Differential Diagnosis of Feline Leukemia Virus Subgroups Using Pseudotype Viruses Expressing Green Fluorescent Protein

Megumi NAKAMURA1,2,3), Eiji SATO1), Tomoyuki MIURA2,3), Kenji BABA3),**, Tetsuya SHIMODA4) and Takayuki MIYAZAWA1)*

1)Laboratory of Signal Transduction, Department of Cell Biology, 2)Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawaracho, Sakyo-ku, Kyoto 606–8507, 3)Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-Nihonnachtsu, Sakyo-ku, Kyoto 606–8501 and 4)SANYO Animal Medical Center, 357–1 Koumoto, Akaia, Okayama 709–0821, Japan.

(Received 13 May 2009/Accepted 19 January 2010/Published online in J-STAGE 2 February 2010)

ABSTRACT. Feline leukemia virus (FeLV) is classified into three receptor interference subgroups, A, B and C. In this study, to differentiate FeLV subgroups, we developed a simple assay system using pseudotype viruses expressing green fluorescent protein (GFP). We prepared gfp pseudotype viruses, named gfp(FeLV-A), gfp(FeLV-B) and gfp(FeLV-C) harboring envelopes of FeLV-A, B and C, respectively. The gfp pseudotype viruses completely interfered with the same subgroups of FeLV reference strains on FEA cells (a feline embryonic fibroblast cell line). We also confirmed that the pseudotype viruses could differentiate FeLV subgroups in field isolates. The assay will be useful for differential diagnosis of FeLV subgroups in veterinary diagnostic laboratories in the future.

NOTE Internal Medicine

Differential Diagnosis of Feline Leukemia Virus Subgroups Using Pseudotype Viruses Expressing Green Fluorescent Protein

Feline leukemia virus (FeLV) is an infectious agent which causes a range of neoplastic and degenerative diseases in cats [3]. The prevalence of FeLV varied by the areas surveyed and the rearing status, and a recent wide-range survey of FeLV infection in Japan revealed that about 2.9% of cats were positive for FeLV antigens in the blood [8]. FeLV isolates are classified into at least three receptor interference subgroups A, B and C [5, 9]. FeLV-A is a transmissible form of FeLV and basically ecotropic [11]. FeLV-B arises by the recombination of FeLV-A with endogenous FeLV in the envelope (env) region in vivo [12, 19]. FeLV-C arises by mutations in the env gene of FeLV-A [16]. Both FeLV-B and FeLV-C have a broad host cell range which includes human cells [5, 12, 14]. FeLV-A is considered to be less pathogenic than other subgroups [12]. FeLV-B induces leukemia in a relatively short period and FeLV-C induces severe anemia [12, 13, 15]. Each subgroup utilizes a distinct receptor and interferes with the same subgroup in the cells, i.e., FeLV can not infect cells previously infected with the same subgroup [7, 11]. The expression of the receptor on the cell surface is hindered by envelope protein (Env) expressed by FeLV which has infected the cells. This phenomenon is called ‘receptor interference’. FeLV isolates can be classified using this characteristic. The frequency of isolation of each FeLV subgroup is different. FeLV-A can be isolated from almost naturally infected cats, whereas FeLV-B is found in half of naturally infected cats and FeLV-C is found very rarely [2, 4, 12]. In a previous study, the isolation rate of FeLV-A alone was 50%, the combination of FeLV-A and FeLV-B was 49%, and combinations of ‘FeLV-A, B and C’ and ‘FeLV-A and C’ was only 1% [2, 4].

In viremic cats, viral antigens are present abundantly in blood; however, the amount of specific antibody against FeLV is very small; therefore, to diagnose FeLV infection, viral antigens are usually detected by a commercial enzyme-linked immunosorbent assay (ELISA) kit [6]. In advanced diagnostic laboratories, FeLV antigens in leukocytes and platelets are also detected by indirect immunofluorescent assay to confirm FeLV infection in myeloid cells [6]. The definitive assay for FeLV infection is virus isolation (VI) [6]. In the VI test, serum samples are inoculated into feline fibroblastic cell lines, such as FEA cells, and the presence of FeLV is confirmed by the focus assay using sarcoma-positive leukemia-negative cells [6, 17]. Although all subgroups can be detected by ELISA and VI tests, they can not be distinguished. For prognosis, it is desirable to differentiate FeLV subgroups in infected cats. To differentiate FeLV subgroups based on receptor interference, murine sarcoma virus (MSV) pseudotyped with each FeLV subgroup is inoculated into sample-inoculated feline fibroblastic cells and the induced foci composed of transformed cells are observed under microscopy [17]. Although this method is reliable, it is rather laborious and requires skill to make foci visible in infected cells. In this study, to differentiate FeLV subgroups, we developed a simple assay system using pseudotype viruses expressing green fluorescent protein (GFP). MSV-pseudotype viruses contain v-mos oncogene and transform the infected cells, whereas gfp pseudotype viruses do not contain oncogenes in viral particles; thus, this
assay is safer than the assay using MSV-pseudotype viruses from the stand point of biohazard issues.

Human embryonic kidney (HEK) 293T cells [18], FEA cells (feline embryonic fibroblast cells) [5, 17] and TELCeB6 (a packaging cell line which expresses large numbers of murine leukemia virus [MLV] core particles incorporating an MFgGnsLacZ vector) [1] were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Platinum-E (Plat-E) cells (a packaging cell line derived from HEK293T cells) [10] were cultured in DMEM supplemented with 10% FCS, blasticidin (10 µg/ml), puromycin (1 µg/ml), penicillin (100 units/ml), and streptomycin (100 µg/ml). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

To prepare stock viruses of FeLV subgroups A, B, and C, we transfected infectious molecular clones of FeLV into HEK 293T cells to avoid recombination with the endogenous FeLV genome present in feline cells [13]. One microgram of each infectious molecular clones of FeLV-A, B and C, named pFGA-5 [19], PGAHF [19] and pFSC [15], respectively, was transfected twice into HEK293T cells using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany). Three days after transfection, culture supernatants were harvested, filtered through a 450 nm membrane filter, and stored at –80°C as stock viruses.

To prepare gfp pseudotype viruses, 0.5 µg each of pMX-GFP (an expression plasmid of MLV) and pCAG-VSV-G (an expression plasmid of vesicular stomatitis virus G protein [VSV-G]) was co-transfected into Plat-E cells using FuGENE6 and then incubated for two days (Fig. 1A). Culture supernatants containing a gfp pseudotype virus harboring VSV-G, termed gfp(VSV-G), were collected and filtered through a 450 nm membrane filter. The gfp (VSV-G) (500 µl culture supernatant) was then inoculated into TELCeB/FBFeLV-A [11], TELCeB/FBFeLV-B [11] and TELCeB/FBFeLV-C cells [11], and cultured in 24-well plates to establish TELCeB/GFP/FBFeLV-A, TELCeB/GFP/FBFeLV-B and TELCeB/GFP/FBFeLV-C cells, respectively (Fig. 1B). Alternatively, to prepare gfp pseudotype viruses, one µg of pMX-GFP was transfected into TELCeB/FBFeLV-A, TELCeB/FBFeLV-B and TELCeB/FBFeLV-C cells grown in six-well plates using FuGENE6 to establish TELCeB/GFP/FBFeLV-A, TELCeB/GFP/FBFeLV-B and TELCeB/FBFeLV-C cells, respectively (Fig. 1B). The culture supernatants of the transformants were collected and filtered through 450 nm membrane filters, and then frozen at ~80°C as stock viruses of gfp pseudotype viruses, named gfp(FeLV-A), gfp(FeLV-B) and gfp(FeLV-C), respectively (Fig. 1B). These pseudotype viruses contain lacZ and gfp genes as viral genomes, the viral core of MLV and Env from each FeLV subgroup.

The gfp pseudotype viruses were titrated on FEA cells, which are susceptible to FeLV-A, B and C. FEA cells were seeded in 24-well plates (5.0 × 10⁵/well) 17 hr before infection. After four-hour infection of serially diluted pseudo-type viruses (200 µl each) in the presence of eight µg/ml polybrene (hexadimethrine bromide) (Sigma-Aldrich, Steinheim, Germany) for viral adsorption, the virus solution was removed and the cells were cultured in growth medium. Two days after infection, the cells were observed under a UV microscope (× 200) and gfp-positive cells were counted. The virus titers were calculated and expressed as infectious units (IU)/ml. The gfp pseudotype viruses were adjusted to 500 IU/ml.

Next, we examined whether gfp pseudotype viruses can be used to differentiate FeLV subgroups in FEA cells which are used widely for the VI test [5, 6]. To prepare FeLV-infected FEA cells, FEA cells were inoculated with either FeLV-A or FeLV-B or FeLV-C or ‘FeLV-A and -B’ in the presence of eight µg/ml polybrene for four hour for adsorption, cultured for two weeks and designated as FEA/FeLV-A, FEA/FeLV-B, FEA/FeLV-C and FEA/FeLV-A+B cells, respectively (Fig. 1C). Then, 100 IU of gfp pseudotype viruses (200 µl) were inoculated into these cells, which were cultured in 24 well-plates. Consequently, gfp(FeLV-A), gfp(FeLV-B) and gfp(FeLV-C) infected naïve FEA cells well (Fig. 2A). gfp(FeLV-A) infected FEA/FeLV-B cells, but neither FEA/FeLV-A nor FEA/FeLV-A+B cells (Fig. 2A). On the other hand, gfp(FeLV-B) infected FEA/FeLV-A cells, but neither FEA/FeLV-B nor FEA/FeLV-A+B cells (Fig. 2A). In addition, gfp(FeLV-C) infected FEA/FeLV-A, FEA/FeLV-B and FEA/FeLV-A+B cells (data not shown), but not FEA/FeLV-C cells (Fig. 2B). From these data, we conclude that gfp pseudotype viruses can be applied to the differential diagnosis of FeLV subgroups A, B and C in FEA cells.

Finally, we applied gfp pseudotype viruses to determine FeLV subgroups in field isolates. FeLV antigen-positive blood samples (2 ml each) were obtained from five cats brought to SANYO Animal Medical Center (Akaiawa, Okayama, Japan) and an FeLV-positive pet cat (named Kotubu) reared in Kyoto, Japan. To isolate FeLV, white blood cells separated from peripheral blood were stimulated with 20 µg/ml concanavalin A (Con-A) for three days and then cultured for an additional seven days in the presence of recombinant human interleukin-2 (IL-2). Con-A and IL-2-stimulated peripheral blood mononuclear cells were cocultured with FEA (for samples from SANYO Animal Medical Center) or HEK293T cells (for Kotubu), and then further cultured for at least two weeks. FeLV isolated using HEK293T cells was transferred to FEA cells and cultured for an additional two weeks. These FeLV isolates were designated as strains SAM-1 to SAM-5 and KTB, respectively (Table 1). Additionally, we obtained FEA cells persistently infected with FeLV strains F8513 and F8701 from Dr. M. Mochizuki (Kyoritsu Seiyaku Co., Tokyo, Japan). These FeLV strains were isolated at Kagoshima University (Kagoshima, Japan) in 1985 and 1987, respectively (Table 1). We then determined the FeLV subgroups of these isolates using gfp pseudotype viruses. Among the eight isolates, five isolates were found to be composed of both FeLV-A and B, but the other three isolates were FeLV-A
Fig. 1. Preparation of a gfp pseudotype virus harboring VSV-G protein (A) and gfp pseudotype viruses harboring FeLV-A, B and C (B). (C) The principle of receptor interference in FEA cells infected with FeLV subgroups. Details are described in the text.

Fig. 2. Infectivity of gfp pseudotype viruses to FEA cells infected with FeLV-A, B and C. FEA, FEA/FeLV-A, FEA/FeLV-B and FEA/FeLV-A+B cells were inoculated with 100 IU of gfp(FeLV-A) and gfp(FeLV-B) (A). FEA and FEA/FeLV-C were inoculated with 100 IU of gfp(FeLV-C) (B). Two days after infection, the cells were observed under UV microscope (×200).
only (Table 1). FeLV-C was not found in the FeLV isolates tested. From these data, we conclude that gfp pseudotype viruses can be applied to differential diagnosis of FeLV in field isolates.

 Taken together, we developed an assay system to differentiate FeLV subgroups using gfp pseudotype viruses. This assay is very simple and can be completed in two days. Once gfp pseudotype viruses are prepared, the viruses can be stored at −80°C for a long period; therefore, the assay will be useful for differential diagnosis of FeLV subgroups in veterinary diagnostic laboratories in the future.

ACKNOWLEDGMENT(S). We thank Dr. M. Mochizuki (Kyoritsu Seiyaku Co., Tokyo, Japan) for providing FeLV strains F8513 and F8701. We are grateful to Dr. Y. Takeuchi (University College London, London, U.K.) for providing TELCeB6 cells. We are also grateful to Prof. J. C. Neil and Prof. O. Jarrett (Glasgow University, Glasgow, U.K.) for providing FEA cells, pFGA-5, pGAHF and pFSC. We thank Prof. T. Kitamura (The University of Tokyo, Tokyo, Japan) for providing FEA cells, pMX-GFP and Plat-E cells. This study was supported by grants from the Ministry of Education, Culture, Science and Sports of Japan and from the Bio-oriented Technology Research Advancement Institution.

REFERENCES


Table 1. Differential diagnosis of FeLV subgroups in field isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Place of sampling</th>
<th>Year of sampling</th>
<th>FeLV subgroups</th>
<th>Diagnosis/Symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM-1</td>
<td>Okayama</td>
<td>2009</td>
<td>A</td>
<td>Immune-mediated pancytopenia</td>
</tr>
<tr>
<td>SAM-2</td>
<td>Okayama</td>
<td>2009</td>
<td>A</td>
<td>Sepsis (immunodeficiency)</td>
</tr>
<tr>
<td>SAM-3</td>
<td>Okayama</td>
<td>2009</td>
<td>A+B</td>
<td>Thymic lymphoma</td>
</tr>
<tr>
<td>SAM-4</td>
<td>Okayama</td>
<td>2009</td>
<td>A+B</td>
<td>Immune-mediated pancytopenia</td>
</tr>
<tr>
<td>SAM-5</td>
<td>Okayama</td>
<td>2009</td>
<td>A+B</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>KTB</td>
<td>Kyoto</td>
<td>2008</td>
<td>A+B</td>
<td>&quot;Leukopenia, Seizure&quot;</td>
</tr>
<tr>
<td>F8513</td>
<td>Kagoshima</td>
<td>1985</td>
<td>A+B</td>
<td>unknown</td>
</tr>
<tr>
<td>F8701</td>
<td>Kagoshima</td>
<td>1987</td>
<td>A</td>
<td>unknown</td>
</tr>
</tbody>
</table>

199: 1362–1364.