hypothesized that DP T cells were more vulnerable to SIV infection than CD4SP T cells. PBMCs, activated by ConA for 24 hr as described above, were cultured in RPMI growth medium for a further 4 days. CD4-positive cells were then enriched from PBMCs using the CD4 microbead MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) to obtain a cell population containing only CD4SP and DP T cells. Flow cytometric analysis confirmed the purity of CD4-positive cells to be 98–99%. Soon after MACS separation, cells were infected with SIVmac239 [6] at a moi of 0.1. A mock control was also prepared in the same manner. At days 3, 5, 7, and 9 post-SIV infection, cultured cells were collected and subjected to flow cytometric analysis to detect a change in the cell population, and CCR5 expression on CD4SP and DP T cells. SIV growth was monitored by the measurement of SIV reverse transcriptase (RT) in SIV-infected cell supernatant. The supernatants were collected and stored at –80°C until used for RT assay. Virion-associated RT was measured by a commercial RT assay (Reverse Transcriptase Assay, colorimetric; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Experiments were performed independently with cells from three individual monkeys (MM132, MM450 and MM452). Results from the RT assay showed that SIV replicated well in cells from MM132 and MM450, and less effectively in those from MM452 (Fig. 2A). The percentage of CCR5-positive cells among the CD4-positive population was low in PBMC from MM452 (data not shown), which may have resulted in less effective SIV replication. On day 9 post-infection, the population of CD4-positive cells decreased in the SIV-infected group as compared to the mock control (Fig. 2B). To determine whether DP T cells were more vulnerable to SIV infection than CD4SP T cells, the percentage of either CD4SP or DP T cells in the SIV-infected group versus in the mock group was calculated at the times indicated in Fig. 2C. In experiments from all three animals, the percentage of residual DP T cells was significantly lower than that of CD4SP T cells at day 9 (Fig. 2C). The efficiency of viral replication was different in each monkey; however, it is important to note that even in the case of poor viral replication, the percentage of residual DP T cells was significantly lower than that of CD4SP T cells. These findings suggest that *in vitro* activated DP T cells were more vulnerable to SIV infection than CD4SP T cells. Although a previous study using herpesvirus saimiri transformed rhesus T cells showed similar results [13], this is the first report using primary PBMCs, and thereby more closely reflects the *in vivo* situation.

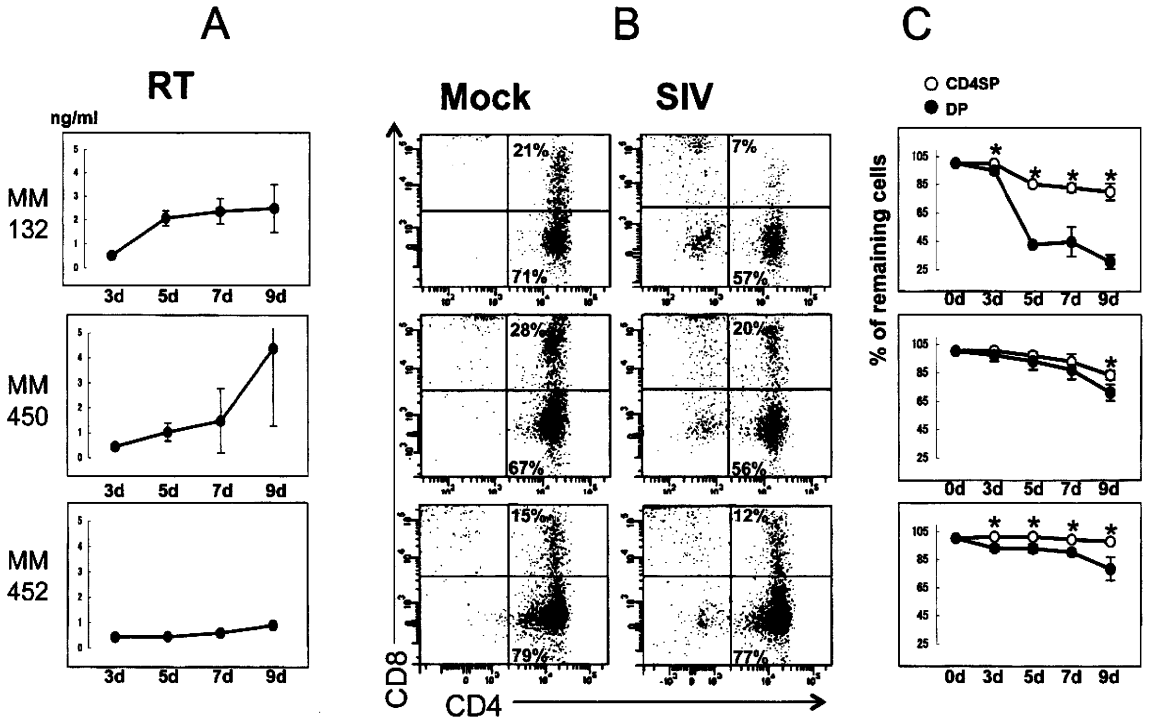

Fig. 2. SIV growth kinetics and populations of CD4SP and DP T cells post-SIV infection in experiments from three individual monkeys. (A) Viral replication was monitored by measurement of RT from the supernatant of SIV-infected cells. Average ± SD from triplicate experiments is shown. (B) Flow cytometric analysis of cell populations of mock and SIV-infected cells. These images show the CD4/CD8 pattern at day 9 after mock or SIV infection. (C) Kinetics of the remaining CD4SP and DP T cell populations post-SIV infection; shown as the percentage of cells in the mock experiment. Average ± SD from triplicate experiments is shown. Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant between DP and SP ($P<0.05$).

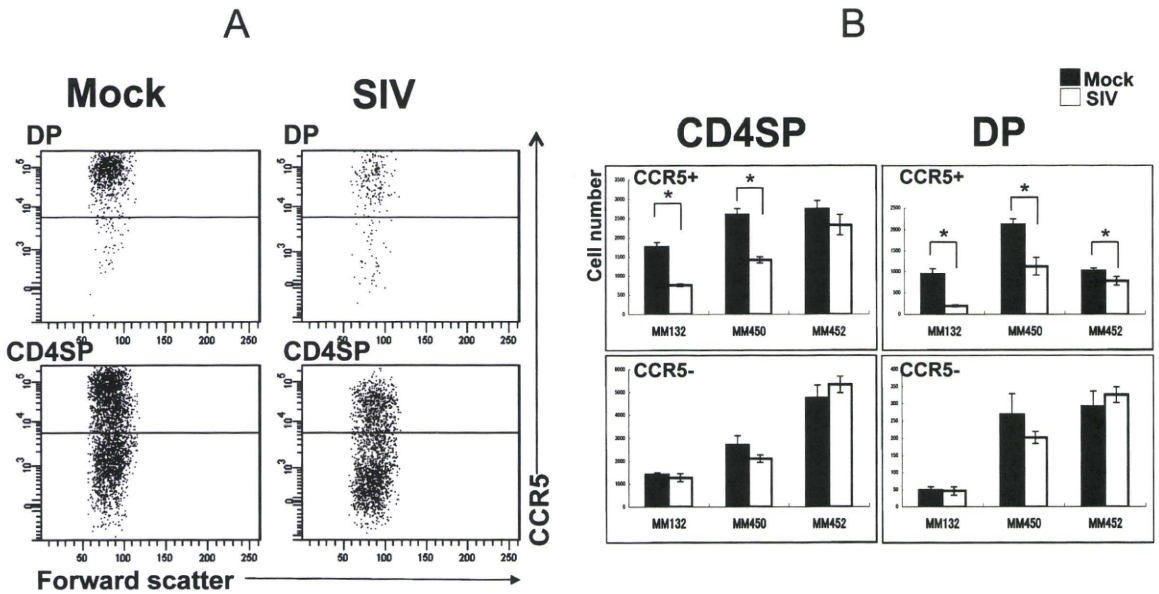

Fig. 3. Change in CCR5-positive cell numbers in CD4SP and DP T cells post-SIV infection. (A) Flow cytometric analysis of CCR5-positive cell populations of mock and SIV-infected cells. The figure shows the pattern of CCR5 expression at day 9 after mock or SIV infection (data from MM132). (B) Comparison of the number of CCR5-positive and CCR5-negative cells in CD4SP and DP T cells between mock and SIV-infected cells. A total of 30,000 events per sample were collected from flow cytometric analysis. Vertical axis is the cell number of each phenotype among all 30,000 events. Average ± SD from triplicate experiments is shown. Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant ($P<0.05$).

To examine the relationship between CCR5 expression and decreased cell counts following *in vitro* SIV infection, the population of cells expressing CCR5 was quantified, in both CD4SP and DP T cells, by comparing SIV-infected cells with the mock control. By flow cytometric analysis, the decrease in CCR5+ cell populations was observed both in CD4SP and DP T cells at day 9 post-SIV infection (Fig. 3A: one of the replicates from MM132). The number of CCR5+ and CCR5- cells was examined in both CD4SP and DP T cell populations by comparing the SIV-infected group to that of the mock group. As for CCR5- cells, no significant decrease was observed in the experiments from all three animals. In contrast, a significant decrease in CCR5+ cell numbers was observed both in CD4SP and DP T cells from two monkeys (Fig. 3B; MM132 and MM450). A similar tendency was observed in the cells from the other animal (MM452). It is possible that CCR5+ cells were selectively depleted both in CD4SP and in DP T cells after SIV infection. While the release of viruses into the cultured fluids from CD4+ selected lymphocytes provides indirect evidence of SIV replication, identification of the actual infected cells in the population would be preferred. Further study is required to clarify this point.

Our present data may indicate that the ability of DP T cells to readily express CCR5 after activation hastens DP T cell death by SIV infection *in vivo*. Naïve lymphocytes recirculate through secondary lymphoid organs where priming occurs, followed by homing to effector sites [3]. Lymphocytes in the bloodstream are activated when they are passing through these lymphoid organs. Thus, our data suggest that DP T cells were more vulnerable to SIV infection than CD4SP T cells, not only in the intestine [14, 15] but also in other tissues containing lymphocytes. In other words, all extrathymic DP T cells in the body were possibly more vulnerable to SIV infection than CD4SP T cells. In fact, an *in vivo* SIV inoculation study showed an abrupt decline in peripheral blood DP T cells at the early stage of infection [1]. Furthermore, the decline in DP T cells was not restored at any point during the infection study, whereas restoration of CD4SP T cells occurred in some monkeys [1]. This may indicate that the deletion of DP T cells in several lymphoid organs is more severe than that of CD4SP T cells after SIV infection.

Although the physiological significance of DP T cells in the body is not fully elucidated, DP T cells are thought to have certain immune functions, including helper and cytotoxic activities [9]. Thus, the systemic depletion of DP T cells in infected animals may correlate to the pathogenicity of SIV.

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NOTE *Internal Medicine*

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

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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.

KEY WORDS: diagnosis, FeLV, leukemia, pseudotype virus, subgroups.

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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 all 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

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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 MFGnlsLacZ vector) [1] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 units/m*l*), and streptomycin (100 μg/m*l*). 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/m*l*), puromycin (1 μg/m*l*), penicillin (100 units/m*l*), and streptomycin (100 μg/m*l*). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 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 each of 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 GFP with a packaging signal 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 μ*l* 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 (5.0×10^4/well) 17 hr before infection. After four-hour infection of serially diluted pseudotype viruses (200 μ*l* each) in the presence of eight μg/m*l* 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)/m*l*. The *gfp* pseudotype viruses were adjusted to 500 IU/m*l*.

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/m*l* 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 μ*l*) 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 m*l* each) were obtained from five cats brought to SANYO Animal Medical Center (Akaiwa, 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/m*l* 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

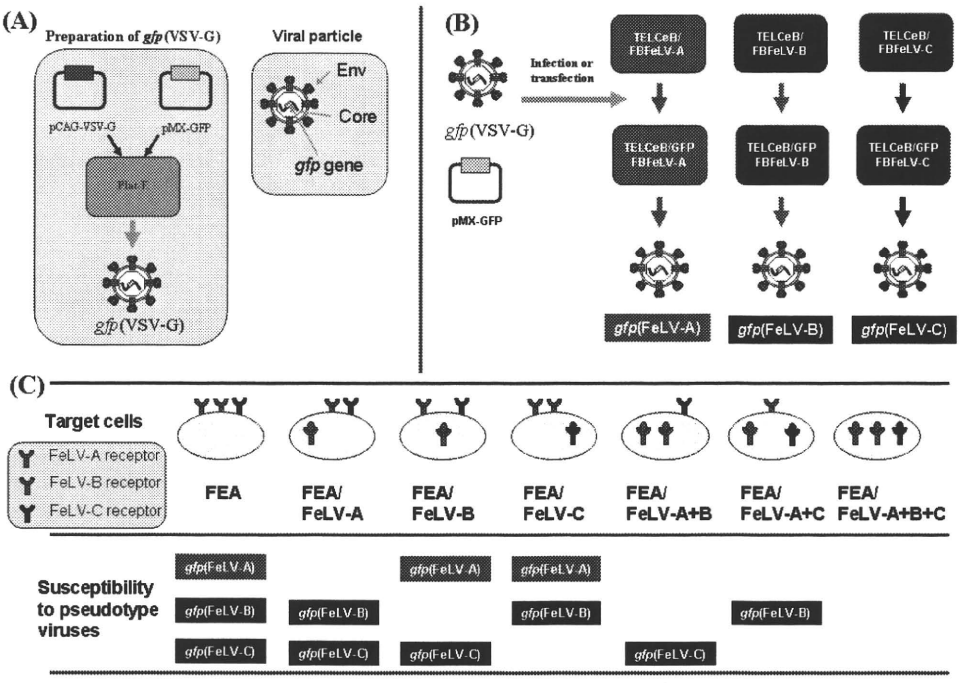

Fig. 1. Preparation of a *gfp* pseudotype virus harboring VSV-G protein (A) and *gfp* pseudotype viruses harboring FeLV-A, B and C (B). (C) The principle of receptor interference in FEA cells infected with FeLV subgroups. Details are described in the text.

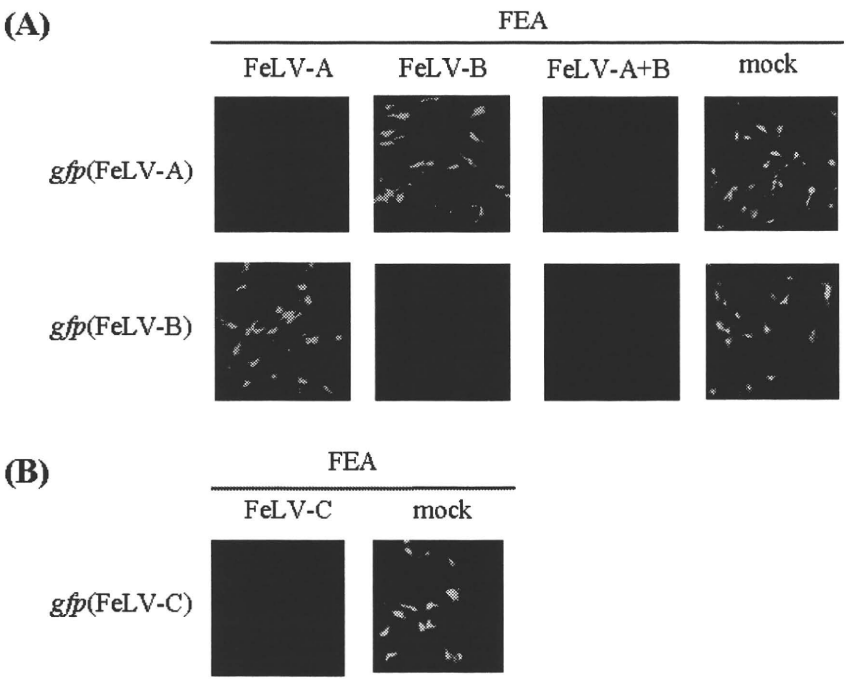

Fig. 2. Infectivity of *gfp* pseudotype viruses to FEA cells infected with FeLV-A, B and C. FEA, FEA/FeLV-A, FEA/FeLV-B and FEA/FeLV-A+B cells were inoculated with 100 IU of *gfp*(FeLV-A) and *gfp*(FeLV-B) (A). FEA and FEA/FeLV-C were inoculated with 100 IU of *gfp*(FeLV-C) (B). Two days after infection, the cells were observed under UV microscope (× 200).

Table 1. Differential diagnosis of FeLV subgroups in field isolates

Strain	Place of sampling	Year of sampling	FeLV subgroups	Diagnosis/Symptom
SAM-1	Okayama	2009	A	Immune-mediated pancytopenia
SAM-2	Okayama	2009	A	Sepsis (immunodeficiency)
SAM-3	Okayama	2009	A+B	Thymic lymphoma
SAM-4	Okayama	2009	A+B	Immune-mediated pancytopenia
SAM-5	Okayama	2009	A+B	Acute lymphoblastic leukemia
KTB	Kyoto	2008	A+B	"Leukopenia, Seizure"
F8513	Kagoshima	1985	A+B	unknown
F8701	Kagoshima	1987	A	unknown

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.

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