Comparative study of the cell tropism of feline immunodeficiency virus isolates of subtypes A, B and D classified on the basis of the env gene V3–V5 sequence

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Feline immunodeficiency virus (FIV) isolates have been classified into subtypes A, B, C and D based on the env gene V3–V5 sequence. The cell tropism of seven new Japanese isolates and a Petaluma (prototype) isolate of FIV, which classified into subtypes A, B and D, for feline lymphoblastoid and feline fibroblastoid cell lines was compared. FeT-1 (CD4+, CD8- and CD9++) and Kumi-1 (CD4++, CD8- and CD9++) cells were used as the interleukin-2 (IL-2)-dependent feline T-lymphocyte cell lines and FeT-J (CD4+, CD8 and CD9+) and 3201 (CD4++, CD8+ and CD9-) cells were used as the IL-2-independent feline T-lymphocyte cell lines. The feline fibroblastoid cell lines used were Crandell feline kidney (CrFK) and fcwf-4 (both CD4-, CD8- and CD9++) cells. All FIV isolates replicated in all lymphoblastoid cell lines used. All isolates showed the greatest cytopathogenicity for Kumi-1 cells. All isolates replicated even in the CD9-negative 3201 cells. More isolates caused persistent infection in IL-2-independent cell lines than in IL-2-dependent cell lines. The number of subtype B isolates that established persistent infection was limited, only one of four strains. Only the subtype A isolates replicated in CrFK cells, whereas none of the isolates replicated in fcwf-4 cells, which have similar cell surface markers to CrFK cells. The subtype A viruses (CrFK/Petaluma, CrFK/Sendai-1) growing in CrFK cells showed greater cytopathogenicity for lymphoblastoid cell lines than did those (FL-4/Petaluma, Kumi-1/Sendai-1) growing in a lymphoblastoid cell line.

Introduction

Feline immunodeficiency virus (FIV) was first isolated from domestic cats with an immunodeficiency-like syndrome in Petaluma, California, USA in 1986 (Pedersen et al., 1987). Based on biological and morphological similarities between FIV and human immunodeficiency virus (HIV), FIV infection in domestic cats is now considered to be an important small-animal model for studying prophylactic and therapeutic strategies against HIV infection (Gardner, 1991; Johnson et al., 1994; Pedersen et al., 1989). As in humans infected with HIV-1, the immunological abnormalities in domestic cats infected with FIV are a chronic and progressive depletion of feline CD4-positive peripheral blood lymphocytes (PBL), a reduction in the CD4:CD8 ratio and, in some cases, an increase in CD8-bearing lymphocytes (Ackley et al., 1990; Barlough et al., 1991; Hoffmann-Fezer et al., 1992; Novotney et al., 1990; Torten et al., 1991). In addition to such changes in the amounts of individual lymphocyte subsets, decreases in lymphocyte blastogenesis responses to concanavalin A, pokeweed mitogen and phytohaemagglutinin, and in primary responses to T cell-dependent antigens were observed (Lin et al., 1990; Siebelink et al., 1990; Taniguchi et al., 1990, 1991; Torten et al., 1991).

FIV is composed of structural genes 5'gag-pol-env 3', flanked by long terminal repeats (LTR), as are other retroviruses (Talbott et al., 1989). Sodora et al. (1994) classified 34 strains of FIV isolated from various places over the world into subtypes A [USA (California) and Europe], B [Japan and USA (not California)] and C (Canada) by comparing the 684 nucleotide sequences (variable regions V3–V5) of the FIV env gene. Recently, we analysed 16 FIV isolates, nine previously reported isolates of A or B and seven new Japanese isolates, based on the nucleotide sequence of gag and the full-length env gene, and classified them into three distinct subtypes, A, B and a new Shizuoka subtype with 20% divergence between pairs of subtypes (Kakinuma et al., 1995). Additional phylogenetic analysis of the V3–V5 regions of 29 worldwide FIV isolates classified them into four major branches: subtypes A, B, C and a new subtype D.

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Based on phylogenetic analysis, our findings establish the existence of Japanese isolates that belong to FIV subtypes A, B and D. The V3–V5 regions of the env gene contain determinants important for cell tropism, cytopathogenicity, infectivity and prominent immunoreactive domains (Lombardi et al., 1993; Pancino et al., 1993; Siebelink et al., 1993). In this paper we compare the cell tropism of the seven new Japanese isolates and the prototype Petaluma strain for feline lymphoblastoid cell lines and feline fibroblastoid cell lines.

**Methods**

**Cell cultures.** FeT-1 and Kumi-1 cells were used as the interleukin-2 (IL-2)-dependent feline T-lymphocyte cell lines. FeT-1 and Kumi-1 cells were cultured in RPMI 1640 growth medium supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics, 50 μg/ml mercaptoethanol, 2 μg/ml Polybrene and 100 U/ml recombinant human IL-2. FeT-J and 3201 cells were used as the IL-2-independent feline T lymphocyte cell lines, and were incubated in the above medium without IL-2. CrFK cells and fcwf-4 cells were used as the feline fibroblastoid cell lines. CrFK and fcwf-4 cells were grown in Eagles' MEM containing 50% Leibovitz's L-15 medium, 10% FCS, 2 μg/ml Polybrene and antibiotics. Of these cell lines, FeT-1, FeT-J, 3201 and CrFK cells were provided by Dr J. K. Yamamoto of the University of Florida, USA. The fcwf-4 cells were provided by M. C. Horznec of the State University Utrecht, The Netherlands. Kumi-1 cells were established from PBL of specific pathogen-free (SPF) cats in our laboratory, and maintained their growth activity over 24 months after cultivation. The Kumi-1 cells could proliferate in culture only in the presence of IL-2.

**Virus isolates.** The seven Japanese isolates of FIV used were Shizuoka, Yokohama, Sendai-1, Sendai-2, Fukuoka, Aomori-1 and Aomori-2, which were isolated by coculturing PBL from cats seropositive for FIV but seronegative for feline leukemia virus with Kumi-1 cells. Among these isolates, Yokohama, Sendai-1, Sendai-2, Fukuoka and Aomori-2 were passaged three times in Kumi-1 cells and used for the experiments. Shizuoka isolates were passaged in Kumi-1 cells four times and Aomori-1 five times before being used. One ml quantities of the supernatant of each isolate growing in Kumi-1 cells were placed in tubes and stored at −80 °C until use. For the FIV prototype Petaluma isolate, the supernatant from cultured FL-4 cells, which are persistently infected with the Petaluma strain, was added to the acetone-fixed cells held at 37 °C for 30 min, they were washed with PBS, mounted in 50% acetone and then subjected to IFA. Serum of an SPF cat experimentally infected with the Petaluma strain was added to the acetone-fixed cells which were then allowed to stand at 37 °C for 30 min. After the specimens were washed with PBS three times, they were stained with FITC-conjugated F(ab)2 of rabbit anti-cat IgG antibody. After being held at 37 °C for 30 min, they were washed with PBS, mounted in 50% glycerol buffer and observed under a fluorescence microscope.

**Polymerase chain reaction (PCR).** For amplification of the p15 region of the gag gene of FIV isolates, the primer 5' GTGATATACCG-AGACCTTA 3' (nucleotides 778–797) was designed as a sense primer, and the primer 5' TTTACTGTITTGATAGGATA 3' (nucleotides 1029–1048) as an antisense primer. The sequences of primers were derived from the sequence of the FIV Petaluma strain (Talbott et al., 1989). PCR was carried out by the method of Hohdatsu et al. (1992). The amplification involved incubation for 5 min at 94 °C as the first denaturation, subsequent cycling which consisted of denaturation for 1 min at 94 °C, primer annealing for 1.5 min at 50 °C and synthesis for 1.5 min at 72 °C, repeated 35 times, and a final incubation for 5 min at 72 °C. The PCR products (10 μl) were analysed by electrophoresis on an 8% polyacrylamide gel. Bands were visualized by ethidium bromide staining and photographed under a UV transilluminator at 312 nm.

**ELISA for FIV antigen.** Each well of ELISA plates coated with MAb to FIV-p24 protein received 100 μl of a sample of the culture fluid, and the plates were incubated at 37 °C for 1 h. After the plates were washed with PBS containing 0.02% Tween 20 (washing solution) four times, an optimal dilution of biotin-labelled cat antibody raised against FIV in PBS containing 10% calf serum and 0.05% Tween 20 (dilution buffer) was added and the plates were incubated at 37 °C for another hour. After another four washes, peroxidase-conjugated avidin diluted with dilution buffer was added to each well. After incubation at 37 °C for 30 min, each well received 100 μl of substrate solution and the plates were incubated at 25 °C for 20 min in a dark room. The reaction was stopped with 1.5 M-H₂SO₄ and the A₄₉₀ was determined.

**Replication and cytopathogenicity of the FIV isolates in feline lymphoblastoid cell lines.** One ml of each of the FIV isolates (about 1000 TCID₅₀) was inoculated onto 5 x 10⁶ Kumi-1 and FeT-1 cells and 2.5 x 10⁶ FeT-J and 3201 cells. After adsorption at 37 °C for 1 h, 5 ml of culture medium was added and the cultures were incubated at 37 °C. Three days later the medium was renewed and all cells were put in the same culture flask for further incubation at 37 °C. Thereafter the medium was renewed in the same way at intervals of 3 days. The number of viable cells was determined at that time by trypsin blue staining. Replication of FIV was based on the detection of FIV antigen in the cells by IFA and the detection of FIV proviral DNA in the cells by PCR.

**Replication of the FIV isolates in feline fibroblastoid cell lines.** One ml quantities of virus (about 1000 TCID₅₀) were inoculated onto cell sheets formed by incubation of 1 x 10⁶ CrFK and fcwf-4 cells for 1 day in a 25 cm² culture flask, followed by adsorption for 1 h at 37 °C. Subsequently, 5 ml of culture medium was added and the culture was incubated at 37 °C. The culture was passaged five times at intervals of 5 days. FIV p24 protein was detected by ELISA in the culture supernatant of each passage, and FIV antigen or proviral DNA in the cells was detected by IFA and PCR.
Fig. 1. Flow cytometric analysis of expression of CD4, CD8 and CD9 markers on lymphoblastoid and fibroblastoid cell lines. FeT-J, 3201, Kumi-1, FeT-1, CrFK and fcwf-4 cells treated with anti-feline CD4 MAb, anti-feline CD8 MAb or anti-human CD9 MAb were analysed by immunofluorescence flow cytometry as described in Methods. ——, Cells treated with HBSS (control); ———, Cells treated with the MAb.
Fig. 2. Replication and cytopathogenicity of the FIV isolates in feline lymphoblastoid cell lines. Kumi-1, FeT-1, FeT-J and 3201 cells were mock (control) infected or infected with Petaluma and seven new Japanese isolates classified into FIV subtypes A, B and D. After inoculation, the culture medium was renewed every 3 days, and the number of viable cells (○) was determined at that time. Replication of FIV was based on detection of FIV antigen by IFA (●).
Results

Detection of cell surface marker by immunofluorescence flow cytometry

The phenotypic profiles of FeT-J, 3201, Kumi-1, FeT-1, CrFK and fcwf-4 cells were determined by flow cytometric analysis using MAbs raised against feline CD4, CD8 and human CD9. The FACS profiles demonstrated that FeT-J cells were CD4+, CD8+ and CD9++; 3201 cells were CD4++, CD8+, and CD9−; Kumi-1 cells were CD4++, CD8− and CD9++; FeT-1 cells were CD4−, CD8− and CD9++; CrFK cells were CD4+, CD8− and CD9++; fcwf-4 cells were CD4−, CD8− and CD9++. (Fig. 1).

Replication and cytopathogenicity of the FIV isolates in feline lymphoblastoid cell lines

Replication of FIV isolates in lymphoblastoid cell lines with different cell surface markers for CD4, CD8 and CD9, i.e. Kumi-1, FeT-1, FeT-J and 3201 cells, was compared. The number of viable cells of the control for each cell line increased gradually after the start of culture, and then remained constant as those cells coexisted with dead cells (Fig. 2). All FIV isolates replicated in every cell line. However, the percentage of FIV antigen-positive cells was changed to a different degree from that of the percentage of cell viability (Fig. 2); all the isolates became positive for FIV antigen, as shown by IFA, and for proviral DNA, shown by PCR, in association with the decrease in cell viability 6 days after inoculation of virus onto Kumi-1 cells. No isolate showing persistent infection was observed in Kumi-1 cells. The subtype A Petaluma and Sendai-1 and the subtype D Shizuoka isolates became positive for FIV antigen and proviral DNA 9 days after inoculation onto FeT-1 cells, and other isolates became positive 18 days after the inoculation. As the isolates became positive for FIV antigen and proviral DNA, the cell viability decreased gradually and the Petaluma isolate alone showed persistent infection. The subtype B Aomori-1 isolate and other isolates became positive for FIV antigen and proviral DNA 9 days after inoculation onto FeT-1 cells, and other isolates became positive 18 days after the inoculation. The proportion of cells showing persistent infection in the IL-2 independent cell lines was larger than in the IL-2 dependent cell lines.

Replication of FIV isolates in feline fibroblastoid cell lines

Replication of FIV isolates in the CD4- and CD8-negative and CD9-positive feline fibroblastoid cell line CrFK and fcwf-4 cells was investigated by IFA, PCR and ELISA. As shown in Table 1, the subtype A Petaluma and Sendai-1 isolates replicated in CrFK cells. The Petaluma isolate, however, was positive for FIV antigen and proviral DNA by IFA and PCR on the first passage and positive by ELISA on the second passage. The Sendai-1 isolate was positive by PCR on the first passage, by IFA on the third passage, and by ELISA on the fourth passage. No FIV antigen or proviral DNA was detected for any other isolate by the fifth passage. There was a distinct correlation between replication in CrFK cells and FIV isolate subtype. None of the FIV isolates including the subtype A isolates replicated in fcwf-4 cells (data not shown).

| Table 1. In vitro replication of the Petaluma strain and seven new Japanese isolates of FIV for CrFK cells |
|---|---|---|---|---|---|
| Subtype | Virus strain | Method | Passage number* |
| A | Petaluma | IFA | + | + | + | + |
| | | PCR | + | + | + | + |
| | | ELISA | − | + | + | + |
| Sendai-1 | IFA | + | + | + | + |
| | PCR | + | + | + | + |
| | ELISA | − | − | + | + |
| B | Aomori-1 | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |
| Aomori-2 | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |
| Sendai-2 | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |
| Yokohama | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |
| D | Fukuoka | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |
| Shizuoka | IFA | − | − | − | − |
| | PCR | − | − | − | − |
| | ELISA | − | − | − | − |

* After inoculation with virus, the cells were passaged five times at intervals of 5 days. FIV antigen or FIV proviral DNA were detected by IFA, ELISA and PCR in the supernatant fluid and cells of each passage. +, FIV antigen or FIV proviral DNA positive; −, FIV antigen or FIV proviral DNA negative. Small; only the Yokohama isolate infected persistently. The proportion of cells showing persistent infection in the IL-2 independent cell lines was larger than in the IL-2 dependent cell lines.
Cytopathogenicity of the FIV isolates grown in CrFK cells for feline lymphoblastoid cell lines

The cytopathogenicity of the Petaluma and Sendai-1 isolates, which grew in CrFK cells, for feline lymphoblastoid cell lines was investigated. For the Petaluma isolate, viruses grown in CrFK cells (CrFK/Petaluma) and in FL-4 cells (FL-4/Petaluma) were inoculated onto 3201, FeT-J, FeT-1 and Kumi-1 cells. For the Sendai-1 isolate, the viruses grown in CrFK cells (CrFK/Sendai-1) and those grown in Kumi-1 cells (Kumi-1/Sendai-1) were inoculated onto 3201 cells. The inoculum of each virus was adjusted to about 1000 TCID$_{50}$/ml, as described in Methods. As shown in Fig. 3, the viruses grown in the CrFK cells showed greater cytopathogenicity than did those grown in the lymphoblastoid cell lines FL-4 and Kumi-1.

Discussion

All FIV isolates replicate in feline PBL which are stimulated with concanavalin A and maintained with IL-2. Also, the Petaluma isolate, the prototype isolate of FIV, grew even in primary feline macrophages (Brunner & Pedersen, 1989), feline tongue cells (Fc3Tg), G355-5 cells derived from feline fetal brain tissue (Talbott et al., 1989) and feline astrocytes (Dow et al., 1990). The Petaluma isolate grew well even in CD4- and CD8-negative CrFK cells, and cells persistently infected with this isolate were also established (Yamamoto et al., 1988). However, the Japanese isolates classified as subtype B, FIV/TM-1 and TM-2, did not grow in CrFK cells (Miyazawa et al., 1989). It has also been reported that some viruses isolated in lymphoblastoid cells could be adapted to grow in CrFK cells only after several passages in the lymphoblastoid cell line (Baldinotti et al., 1994). All seven new Japanese isolates used in this study have been passaged in a lymphoblastoid cell line three to five times. Of these isolates, those which were classified as subtypes B and D did not grow in CrFK cells, the same as the TM-1 and TM-2 isolates. Only the Sendai-1 isolate classified as subtype A replicated in CrFK cells in the same way as the Petaluma isolate does. Osborne et al. (1994) have recently adapted three isolates, FIV/Glasgow-8, FIV/Amsterdam 4 and 6, which had
been isolated and grown in IL-2-dependent feline T cell cultures, to growth in CrFK cells and used them for cross-neutralization testing between the FIV isolates. These three isolates are also classified as subtype A viruses. It has been reported that some isolates of subtype A FIV show no replication in CrFK cells (Phillips et al., 1990). However, the isolates of at least subtypes B and D did not replicate in CrFK cells in this study. Thus, the ability of FIV to grow in CrFK cells may be considered to be correlated with the subtype. The subtype A viruses (CrFK/Petaluma, CrFK/Sendai-1) growing in CrFK cells showed greater cytopathogenicity for lymphoblastoid cell lines than did those (FL4/ Petaluma, Kumi-1/Sendai-1) grown in lymphoblastoid cell lines. These results suggest that the cell tropism of the virus varies with the cell used for propagation even if the virus strain is constant. At present the reason why the viruses grown in CrFK cells showed strong cytopathogenicity for lymphoblastoid cell lines is not known.

Osborne et al. (1994), who compared the sequence of the env V3 region of the viruses grown in lymphoblastoid cell cultures and those adapted and grown in CrFK cells, reported the absence of change in either of the two types of virus. Some studies have shown the possibility that the env V3 region determines cell tropism in HIV and simian immunodeficiency virus (Cann et al., 1992; Hwang et al., 1991; Kirchhoff et al., 1994).

In fcwf-4 cells that have CD4-, CD8- and CD9++ cell surface markers similar to those of CrFK cells, none of the isolates grew, including the Petaluma isolate. Although identification of the cellular receptor of FIV is still uncertain, CD4 and CD9 molecules are under investigation as candidates (Brown et al., 1991; Hosie et al., 1993; Miyazawa et al., 1992; Siebelink et al., 1992; Tokunaga et al., 1992; Willett et al., 1991, 1994). The number of CD4-positive lymphocytes decreases in FIV infection in a way similar to HIV infection, suggesting that CD4 molecules play an important role as virus receptors (Ackley et al., 1990; Barlough et al., 1991; Hoffmann-Fezer et al., 1992; Novotney et al., 1990; Torten et al., 1991). However, Norimine et al. (1993) reported that the subtype B FIV/TM-1 and TM-2 did not grow in CrFK cells even though CD4 molecules were expressed and that CD4 alone does not function as a virus receptor. Willett et al. (1994) suggested that CD9 molecules rather than CD4 molecules are important as the cellular receptor of FIV. Hosie et al. (1993) have reported that the Glasgow-8 strain of subtype A does not grow in CD4-positive and CD9-negative 3201 cells. However, we found that all the FIV isolates grew in 3201 cells, in contradiction to the results of Hosie et al. (1993) and Willett et al. (1994). Tochikura et al. (1990) also reported growth of FIV in 3201 cells. The Kumi-1 cells in which the strongest cytopathogenicity was observed were strongly positive for both CD4 and CD9 molecules. Subtype A isolates replicated in CD4-negative and CD9-positive CrFK cells, but no subtype B or D isolates did. None of the FIV isolates replicated in CD4-negative and CD9-positive fcwf-4 cells. Thus, there was no correlation between the cell surface antigens of the cell lines used in this study and the susceptibility to specific FIV isolates. From these observations, we consider it unlikely that either CD4 or CD9 function as the sole cellular receptor for FIV.

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