Arch Virol (1993) 130: 171–178

Archives of Virology

Feline CD 4 molecules expressed on feline non-lymphoid cell lines are not enough for productive infection of highly lymphotropic feline immunodeficiency virus isolates

Brief Report

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Accepted October 14, 1992

Summary. To investigate whether the feline CD 4 (fCD 4) molecules are involved in infections of highly lymphotropic feline immunodeficiency virus (FIV) isolates, we expressed fCD 4 stably on Crandell feline kidney cells and *Felis catus* whole foetus 4 cells by transfection of a cDNA encoding the fCD 4 glycoprotein, and then infected them with TM 1 and TM 2 strains of FIV, which are unable to infect these cells productively. In spite of fCD 4 being expressed on these cells, no virus production was observed. This result indicates that fCD 4 expression alone cannot induce a productive infection of the FIV TM 1 and TM 2 strains.

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The CD 4 molecule is a major receptor for human immunodeficiency virus type 1 (HIV-1) [6, 12, 13, 15], but the other molecules are also implicated in CD 4-independent infection [3, 7, 8, 26, 30]. Feline immunodeficiency virus (FIV) is an etiological agent of the acquired immunodeficiency-like diseases in cats [25]. Whether the FIV utilizes feline CD 4 (fCD 4) molecules as a receptor or not is unknown at present. However, the decrease of fCD 4/feline CD 8 (fCD 8) T cell ratio after experimental infection of cats with FIV was reported [2, 23]. In addition, our FIV isolates, TM 1 and TM 2 strains can productively infect

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fCD4$^+$ fCD8$^-$ MYA-1 cells but not fCD4$^-$ fCD8$^-$ FL74 cells [14, 17–20]. There are some other Japanese isolates which are also infectious to fCD4$^+$ fCD8$^-$ cell line (Fel-039 cells) [31]. These results imply that fCD4 is one of the candidate molecules for the FIV receptor. On the other hand, it has been reported that both CD4$^+$ and CD8$^+$ cells were productively infected with FIV in vitro [4]. Furthermore, Yamamoto et al. [32] reported that Crandell feline kidney (CRFK) cells were also productively infected by a Petaluma strain of FIV. However, we could not detect any virus production in fCD4-negative CRFK and *Felis catus* whole foetus 4 (fcwf-4) cells by infection with cell-free FIV TM1 or TM2 strain. These contradictory results may be due to the difference in the host range between these virus strains.

To clarify the role of fCD4 on FIV infection, we used feline CD4 cDNA, termed as FT121 which had been cloned in a previous study [21]. In the present study, we established CRFK and fcwf-4 cells on which fCD4 molecules were stably expressed by transfection of the expression plasmid containing FT121 and selective marker gene. The purpose of the present study is to examine the role of fCD4 on infection of FIV TM1 and TM2 strains, using these fCD4 expressing cells.

CRFK [5] and fcwf-4 [9] cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum (FCS) and antibiotics. MYA-1 cells were cultured in RPMI 1640 growth medium supplemented with 10% FCS, antibiotics, 50 μM 2-mercaptoethanol, 2 μg/ml polybrene and 100 units/ml of recombinant human interleukin-2 at 37 °C in a humidified atmosphere of 5% CO_2 in air. For preparation of virus stocks of FIV TM1 and TM2 strains, MYA-1 cells were infected with a low passaged FIV TM1 or TM2 strain, and then the supernate was harvested after 8 day-incubation when the virus titer reached a plateau [11]. These stock viruses were passed through a 0.45 μm Milipore filter and stored at −80 °C in 1 ml aliquots until use. The titration of the stock viruses was carried out as described previously [11].

To express the fCD4 (FT121) in CRFK and fcwf-4 cells, we chose Rous sarcoma virus (RSV) long terminal repeat (LTR) for the promoter of the gene because of the strong activity of the RSV LTR in the cells [22]. *Eco* RI-linked FT121 was blunted and inserted into blunted Hin dIII site of pRVSVneo [28], which contained *neo* resistant gene under the control of simian virus 40 early gene promoter. This expression plasmid was designated as pRVSVfCD4neo. The construction of the plasmid was shown in Fig. 1.

To establish the CRFK and fcwf-4 cells which stably express the fCD4, CRFK, and fcwf-4 cells grown in six-well dishes were transfected with 5 μg of pRVSVfCD4neo DNA by the phosphate calcium coprecipitation method [28]. Two days after transfection, the cell medium was replaced with the one containing 200 μg/ml G418 (Geneticin; Gibco, BRL Life Technologies Inc., Gaithersburg, U.S.A.). The selected cells were maintained in the medium with the same concentration of G418. For examination of fCD4 expression, the cells

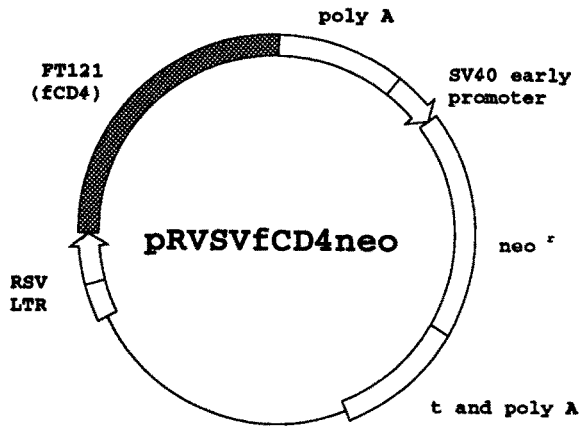

Fig. 1. The construction of the stable expression plasmid, pRVSVfCD4neo. *Eco* RI-linked fCD4 cDNA (FT121) was blunted and inserted into blunted Hin dIII site of pRVSVneo. *poly A* SV40 poly (A); *t* SV40 small t intron; *neo*^{r} *neo* resistant gene

were harvested with phosphate-buffered saline (PBS) containing 2 mM EDTA, washed twice in cold PBS, and resuspended at a concentration of 10^7 cells/ml in ice-cold PBS containing 0.1% sodium azide and 3% FCS. The cells were reacted with the anti-fCD4 monoclonal antibody, termed Fel 7 [1], for 30 min on ice and washed three times in PBS. Indirect stains were visualized with a rabbit anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate for 30 min on ice. After three times of washing in PBS, stained cells were partially analyzed with fluorescence microscopy and a CytoACE cytofluorometer (Japan Spectroscopic Co., Tokyo, Japan). After the G418 selection for over 2 months, the populations of fCD4 positive CRFK and fcwf-4 cells transfected with the pRVSVfCD4neo were 10.3% and 10.0%, respectively (Fig. 2). The fCD4-expressed CRFK and fcwf-4 cells were referred to Cf4 and ff4 cells, respectively. As shown in Fig. 2 (flow cytometric analysis) and Fig. 3 (indirect immunofluorescence assay, IFA), the efficiency of fCD4 expression varied in each cell. This variation was thought to have been caused by the heterogenous population of the cells, because these cells had not been cloned. Maddon et al. reported the HeLa cells stably expressing CD4, which were permissive for productive infections of HIV-1 [13]. In this case, the cells were cloned cells and CD4 expression was very high. We did not clone the transfected cells, however, the expression efficiency of fCD4 as observed in Cf4 and ff4 cells seemed to be sufficient for the infection assay for FIV, as long as the virus uses fCD4 efficiently as the receptor.

Next, we examined the virus susceptibility of these cells. CRFK, fcwf-4, Cf4 and ff4 cells were infected with FIV TM1 or TM2 strain at an moi of 0.3 $TCID_{50}$. The cells were passaged by 0.2% trypsin treatments and washed every 6 or 7 days. Twenty days after inoculation, the culture supernate was inoculated to MYA-1 cell cultures for the virus rescue. Simultaneously, these cells were cocultured with MYA-1 cells. Twenty day-incubation period was enough to avoid residual virus contamination from the inoculum used, because these isolates were known to be completely inactivated within a 14-day incubation at 37 °C as previously reported [10]. The MYA-1 cells were periodically ex-

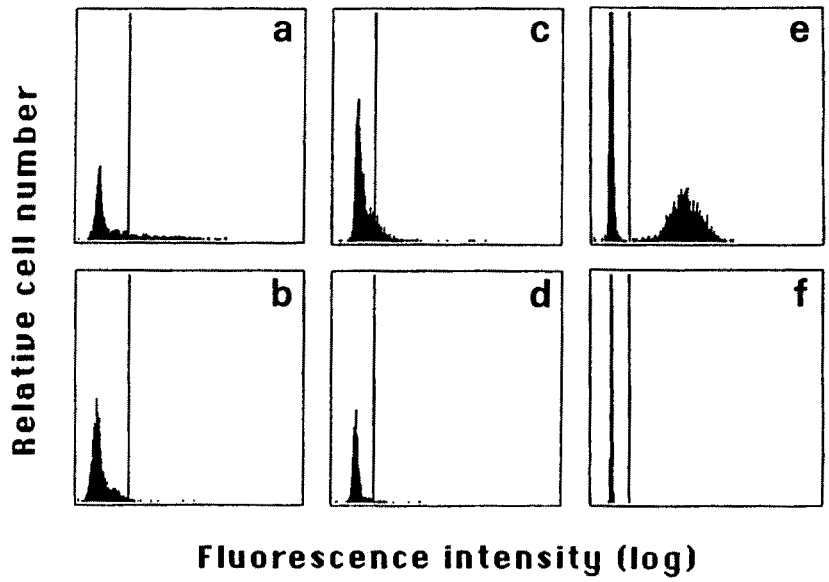

Fig. 2. Flow cytometric analysis. **a** Cf4 cells, **b** CRFK cells, **c** ff4 cells, **d** fcwf-4 cells, **e** and **f** MYA-1 cells. Cells were reacted with the anti-feline CD4 monoclonal antibody (Fel7) (**a**–**e**) or normal mouse IgG (**f**) for 30 min on ice and washed three times in PBS. Indirect stains were visualized with a rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate for 30 min on ice

amined for virus production by the IFA [16, 17] and the reverse transcriptase (RT) assay [24]. Though this experiment was repeated more than 5 times, we could not detect any evidence for virus production of FIV TM 1 or TM 2 strain (data not shown).

Furthermore, we investigated the possibility of the cDNA synthesis of FIV in these cells. For detection of viral cDNA, we used the polymerase chain reaction (PCR) method using the primer flanking the part of *gag* gene of FIV. To avoid the viral DNA contamination, stock virus was treated with 200 μg/ml of DNase (Boehringer Mannheim Yamanouchi, Co., Tokyo, Japan) for 1 h at 37 °C before inoculation. For virus infection, the cells were incubated with the DNase-treated virus for 15 h and washed in PBS three times. Five days after infection, FIV inoculated cells were harvested, and the cells were washed in PBS, lysed in proteinase K buffer (100 mM Tris-HCl pH 7.5, 12.5 mM EDTA, 150 M NaCl, 1% SDS, 200 μg/ml proteinase K) and then subjected to phenol-chloroform extraction and ethanol precipitation. The resultant total DNAs were subjected to PCR amplification.

For amplification of a *gag* fragment of FIV TM 1 and TM 2 strains, the primer
5′-CTGGTGATCCTACTTCTTGGCAGGC-3′, nucleotides 1687–1663,
was designed as an antisense primer, and the primer
5′-CTAGGAGGTGAGGAGGTCCAACTGTG-3′, nucleotides 1126–1151,

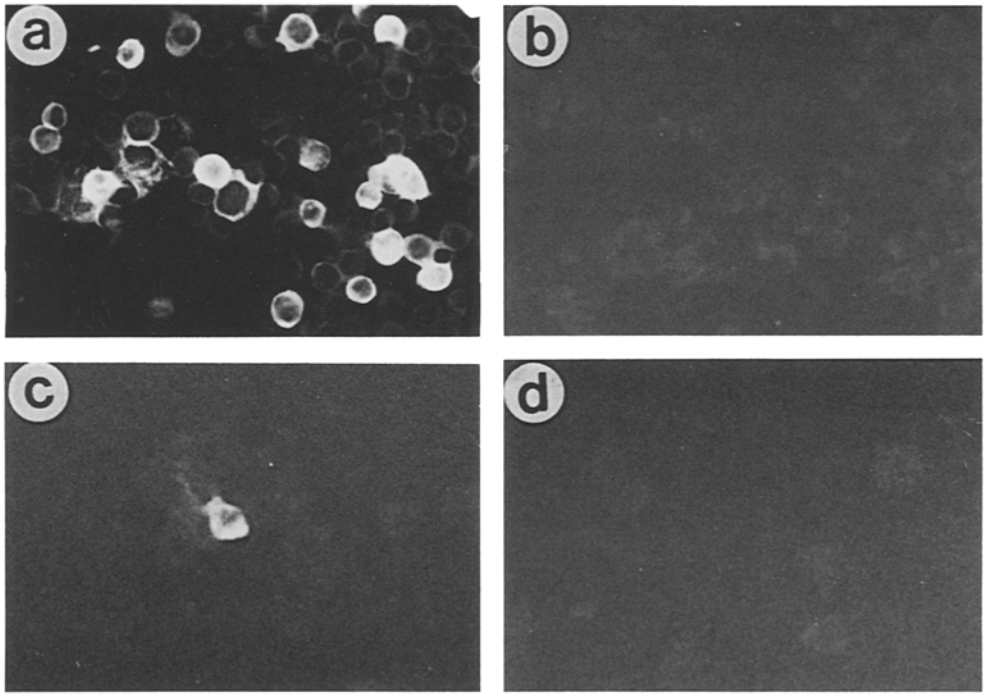

Fig. 3. Indirect immunofluorescence assay. **a** Cf 4 cells, **b** CRFK cells, **c** ff 4 cells, **d** fcwf-4 cells. Cells were reacted with the anti-feline CD 4 monoclonal antibody (Fel 7), and then with a α-mouse IgG conjugated with fluorescein isothiocyanate

as a sense primer. The sequences of primers were derived from the sequence of FIV TM 2 strain [14]. PCR was carried out by the method of Saiki et al. [27] in a 50 µl volume overlaid with an equal volume of mineral oil. A GeneAmp PCR Reagent kit (Perkin Elmer Cetus, Norwalk, U.S.A.) was used for the reactions. Amplification proceeded for 30 cycles in a Thermal Cyclic Reactor Model TC-100 (Hoei Science Co., Tokyo, Japan). One cycle consisted of incubations at 94, 58, and 72 °C for 1, 1, and 2 min, respectively. After amplification, 10 µl of the 50 µl-reaction were electrophoresed on a 2% agarose gel (in Tris-borate-EDTA buffer). Fractionated DNA was transferred to a nylon membrane, and cross-linked by UV. Hybridization was carried out for 18 h at 50 °C in a solution containing 50% formamide, 6 × SSC (1 × SSC: 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS, 5 × Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and the ^{32}P-labelled probe DNA. The amplified DNA from TM 2 clone was used as a probe DNA.

Figure 4 shows the results of the PCR analysis. The cDNA of FIV *gag* gene was detected in the MYA-1 cells infected with FIV TM 1. However, we could not detect any positive band in the CRFK, fcwf-4, Cf 4 and ff 4 cells infected with the virus. Similar results were obtained for the FIV TM 2 (data not shown).

The data obtained in this study revealed that FIV TM 1 and TM 2 strains cannot infect either of the cells irrespective of the fCD 4 expression on the cells.

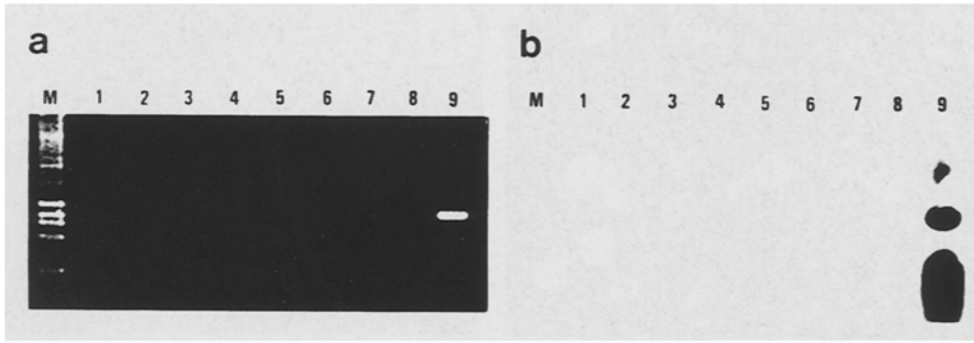

Fig. 4. The amplified PCR products using FIV *gag* primers. FIV *gag*-specific sequences are amplified in all samples. **a** 2% agarose gel stained with ethidium bromide, and **b** Southern blot hybridization. *1*, *5* CRFK cells; *2*, *6* fcwf-4 cells; *3*, *7* Cf4 cells; *4*, *8* ff4 cells; and *9* MYA-1 cells. *1–4* Mock-infected and *5–9* FIV TM 1-infected. *M* λ DNA digested by *Pvu*II as a size marker

Therefore, we concluded that fCD4 expression on CRFK and fcwf-4 cells is not sufficient for the induction of a productive infection of FIV TM 1 and TM 2 strains. An FIV DNA clone containing a full length of TM 1 or TM 2 strain produces the virus in CRFK and fcwf-4 cells by gene-transfection [14, 19; Miyazawa et al., unpubl. data]. Therefore, we considered that the failure of FIV TM 1 and TM 2 strains to infect the cells was due to the blockage at an early stage of infection, such as the viral adsorption, penetration, and cDNA synthesis. From our data, it is unlikely that fCD4 alone is involved in the infection of highly lymphotropic FIV such as TM 1 or TM 2 isolate, and it is likely that some other molecules participate in virus-cell interaction, in addition to fCD4. However, at the moment we cannot exclude the possibility that the FIV TM 1 and TM 2 strains can penetrate in the cells and the incomplete cDNA synthesis of the virus occurs as reported in the case of the HIV-1 infection in stationary cells [29] or quiescent primary lymphocytes [33, 34].

Acknowledgements

We are grateful to Dr. A. Adachi (Kyoto University, Kyoto, Japan) for providing pRVSVneo and we thank Drs. Momoi and Tsujimoto (The University of Tokyo, Tokyo, Japan) for supplying *gag* primers. This study was supported in part by grants from the Ministry of Education, Science and Culture, and from the Ministry of Health and Welfare of Japan. J. Norimine was partly supported by the Supporting Organization of JOCV. T. Miyazawa and Y. Kawaguchi are supported by the Recruit Scholarship.

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Received August 26, 1992