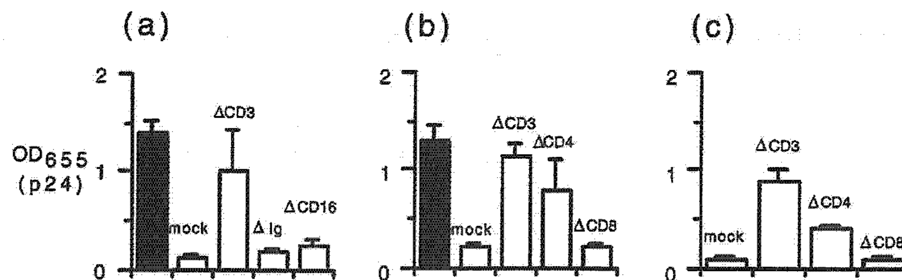

Fig. 3. Effects of depletion of other subpopulations in cat 303 PBLs on FIV replication. By the panning method, the PBMC subpopulations indicated were depleted from PBLs of cat 303 (for example, "ΔCD3" means CD3ε-depleted PBLs, and "mock" means PBLs labeled with no mAb for panning). These depleted cells were mixed with FIV-14-infected (a,b) or uninfected (c) MYA-1 cells and cultured. p24 antigens in culture supernatants were measured by ELISA at day 9 (a,b) or at day 12 (c). Black bars are results in infected MYA-1 cell culture without mixing of the depleted cells. Results are shown as the mean ± S.D. of quadruplicate data. The background (uninfected MYA-1 cell culture alone) is approximately 0.1 OD_{655}.

out. As shown in Fig. 3b, a p24 increase was observed in both CD3ε- and CD4-depleted, but not CD8α-depleted PBLs co-cultured with FIV-14-infected MYA-1. In the last experiment, uninfected MYA-1 was also used for the co-culture, and a significant increase in endogenous p24 was observed in the CD3ε-depleted cell culture (Fig. 3c). In addition, although not significant ($OD_{655} < 0.5$), an increase in p24 was observed in the CD4-depleted cell culture compared with the mock-treated cell culture. Hence, cat 303 certainly had infected cells in the periphery, and $CD4^+$ lymphocytes might be the principal effector hindering FIV replication in PBLs. The non-proliferation of endogenous or exogenous virus in cultures of CD8-depleted PBLs was due to this potent antiviral activity of $CD4^+$ lymphocytes. However, $CD8^+$ lymphocytes also might have weak antiviral activities in this cat, because CD3ε depletion (CD4 and CD8 double depletion [42]) induced more viral replication from an FIV-14-infected cell line than CD4 depletion (Fig. 3b, endogenous FIV replication was negligible, data not shown). These results suggest that more than two distinct populations in one individual can be involved in the suppression of FIV replication.

No replication of virus in CD8-depleted PBMCs of infected individuals, as in the case of cat 303, was occasionally reported in FIV [10,18] and HIV [43] studies. Several reasons for the phenomenon could be proposed; however, detailed immunological analyses for these individuals have not been done. We consider that $CD4^+$ PBLs, although less frequent than $CD8^+$ PBLs, can control lentivirus replication and that the infected individuals described above might control FIV or HIV replication by $CD4^+$ lymphocyte-mediated cytotoxicity [44–47], secretion of interferon-γ and β chemokines [48,49] or other unidentified mechanisms. The lack of a correlation between $CD8^+$ cell numbers and non-cytolytic activities [6,10,18] may be explained by the activity of these CD8-negative lymphocytes. Studies with larger numbers of cats will be needed to test this hypothesis.

3.4. CD4:CD8 ratios

FIV infection induces the decrease of CD4:CD8 ratios of PBLs of the infected cats, and the decreases are often used to estimate immunological disorders [2,4,27,31]. The CD4:CD8α ratios of the cats used in this study were as follows: cat 301, 0.77; cat 302, 0.59; cat 303, 0.33; cat 304, 0.95; cat 305, 0.59; cat 306, 0.77. Three out of five FIV-infected cats showed lower ratios than that of the uninfected one (cat 306). There seemed to be no apparent correlation between the CD4:CD8α ratios and the increases in CD8β-decreased subpopulations within $CD8^+$ PBLs or lymphocyte phenotypes responsible for inhibition of FIV replication. Surprisingly, cat 303, whose $CD4^+$ PBLs showed the potent antiviral activity (Fig. 3), had the lowest CD4:CD8α ratio among the cats.

3.5. Reconstitution assay

To further analyze the antiviral activities, a reconstitution assay was performed based on the results obtained from the depletion assay. $CD8\alpha^+$ cells from cats 301, 304 and 305 and $CD4^+$ cells from cat 303 were isolated by panning from each animal as effector cells that were regarded to suppress FIV replication. The purity of the effector cells for CD8α or CD4 was over 80% (data not shown). PBMCs depleted of the effector cells served as target cells where FIV could proliferate. Mock-treated PBMCs of cat 302 and FIV TM2- or FIV-14-infected MYA-1 cells were also used as target cells. These effector and target cells were mixed in all combinations, cultured, and then measured for p24 antigens in culture at day 12 or 9 (Table 1). Effector cells from cats 301, 303 and 305 inhibited p24 production from self PBMCs significantly (>50% inhibition), confirming the results in the depletion assay, while those from cat 304 did not. The failure of inhibition in cat 304 might be due to a technical error(s) which occurred during preparation of effector cells. Effectors from cats 301, 303 and 305 also inhibited FIV proliferation in non-self targets and in homologous (TM2) or heterologous (FIV-14) FIV strain-infected MYA-1 cells, although not in all combinations. These results suggest that the antiviral activity of $CD4^+$ as well as $CD8^+$ lymphocytes was mediated in a non-restricted manner by MHC, and possibly in an antigen-non-specific manner. No restriction of the activity to homologous isolates was reported [14]. The reason for the absence of suppressive activity of all effectors tested against viral replication in cat 302 PBLs is unclear.

Table 1
Reconstitution assay: co-culture of effector cells with self- or non-self-target cells from FIV-infected cats or with an in vitro-infected cell line (MYA-1)

		Inhibition (%) [a]						
					Target cell			
		Cat301	Cat302	Cat303	Cat304	Cat305	TM2-infected	FIV-14-infected
Effector		ΔCD8α [b]	Whole	ΔCD4	ΔCD8α	ΔCD8α	MYA-1 [c]	MYA-1
Cat301	CD8α [d]	74	15	99	21	95	61	16
Cat303	CD4	97	8	98	100	95	54	84
Cat304	CD8α	33	23	−19	46	43	29	19
Cat305	CD8α	94	−7	32	84	71	48	46

[a] Inhibition percentages were calculated as described in Section 2. More than 50% is regarded as significantly positive.
[b] CD8α-depleted PBLs of cat 301.
[c] MYA-1 cells infected with FIV strain TM2 at an MOI of 0.01.
[d] $CD8\alpha^+$ cells isolated from cat 301 PBLs.

In conclusion, we have demonstrated that various subpopulations of PBLs in FIV intravaginally infected cats ($CD8\alpha^+\beta^+$, $CD8\alpha^+\beta^-$, and $CD4^+$ phenotypes) are involved in the suppression of FIV replication, at least in part, in an MHC-non-restricted manner. A detailed understanding of immune responses in mucosal infection may help with vaccine or antiviral drug development against both FIV and HIV; therefore, further analysis of these cells carrying antiviral activities is important.

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1), rendering this loop more mobile; glutamate brings a further negative charge into the channel on the periplasmic side of the eyelet and may therefore change the permeation properties slightly. The exchange D91G of MspD removes the side chain defining the pore eyelet diameter (Fig. 4B) and should therefore increase the permeability decisively. N102D of MspD introduces a negative charge on the periplasmic side of the eyelet similar to an exchange in MspC. S103T and V104I should improve the stability of the chain at the goblet base, and the glutamine of A96Q projects into the periplasm. I68V is in the nonpolar core of the rim domain, whereas L8V and G141A strengthen the interface between these domains. Exchanges G1A, L2V, E5Q, W21A, D22E, K47T, and I49H are on the outer-domain surface about 20 Å below the goblet rim, varying the charge distribution and thus the expected interaction with components of the outer leaflet of the outer membrane. In particular, the removal of the protruding tryptophan should reduce the sugar-binding capacity (*29*). S136K and E139K on the rim change the antigenic properties of MspD compared with the other isomers. The interactions of MspA with the outer membrane may be analyzed in the crystal because the outer surface of four of the eight rim domains is freely accessible so that soaking experiments with membrane constituents are feasible.

Among the mycobacteria, *Mycobacterium tuberculosis* is carried by about two billion people and causes a higher death rate than any other infectious agent (*30*). *M. tuberculosis* and its close relative *Mycobacterium bovis* have porins with conductances of about 0.8 and 4 nS in 1 M KCl solutions that were detected in detergent extracts but could not be further characterized (*31*, *32*). These porins are thought to be crucial for drug efficacy because the three important drugs—isoniazide, ethambutol, and pyrazinamide—are small polar molecules (*6*) that, for instance, can easily permeate the channel constriction of MspA (Fig. 4B). Because porin sequences are highly variable (*33*), the lack of sequence similarity does not exclude a structural relationship to MspA. Therefore, it seems worthwhile to perform an MspA-based structure knowledge-directed search to identify the large porin in the *M. tuberculosis* genome. Any detected gene may then be expressed, purified, and crystallized like MspA.

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23. Experimental data are available as supporting material on *Science* Online.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
25. The shear number S of a β barrel is derived by running from a given residue of a given β strand along the hydrogen bonds once around the barrel and counting the offset number of residues on return. For a regular 16-stranded β barrel, the square of the circumference is $(4900 + 11S^2)$ Å^2, showing the dependence of the diameter on the shear number (*21*).
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34. The coordinates and structure factors are deposited in the Protein Data Bank under accession code 1UUN. We thank the teams of the European Molecular Biology Laboratory (EMBL)–outstation at Deutsches Elektronen-synchrotron (DESY) (Hamburg) and of the Swiss Light Source (SLS) (Villigen) for their help in data collection; and D. Frey, C. Heinz, P. Hülsmann, D. Kloer, and M. Ziegler for help at various stages of the analysis. Supported by the Deutsche Forschungsgemeinschaft under SFB-388 and Ni-412.

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SOM Text
Fig. S1
Tables S1 and S2
References

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Use of CD134 As a Primary Receptor by the Feline Immunodeficiency Virus

Masayuki Shimojima,[1]* Takayuki Miyazawa,[2,3]* Yasuhiro Ikeda,[4] Elizabeth L. McMonagle,[5] Hayley Haining,[5] Hiroomi Akashi,[1] Yasuhiro Takeuchi,[4] Margaret J. Hosie,[5] Brian J. Willett[5]†

Feline immunodeficiency virus (FIV) induces a disease similar to acquired immunodeficiency syndrome (AIDS) in cats, yet in contrast to human immunodeficiency virus (HIV), CD4 is not the viral receptor. We identified a primary receptor for FIV as CD134 (OX40), a T cell activation antigen and costimulatory molecule. CD134 expression promotes viral binding and renders cells permissive for viral entry, productive infection, and syncytium formation. Infection is CXCR4-dependent, analogous to infection with X4 strains of HIV. Thus, despite the evolutionary divergence of the feline and human lentiviruses, both viruses use receptors that target the virus to a subset of cells that are pivotal to the acquired immune response.

The primary event in the process of viral entry into a target cell is the interaction between the virus and its cellular receptor, and the specificity of this interaction determines both viral cell tropism and pathogenicity. For the primate lentiviruses, the viral receptor is CD4, targeting the virus to helper T cells, resulting in their depletion and the eventual development of acquired immunodeficiency syndrome (AIDS) (*1*). However, CD4 expression alone is insufficient to confer susceptibility to infection with human immunodeficiency virus (HIV), which also depends on the expression of coreceptors, principally the chemokine receptors CXCR4 and CCR5 (*2*). The virus attaches via a high-affinity interaction with CD4, resulting in a conformational change in the envelope glycoprotein (Env) and exposing the binding site for the chemokine receptor (*3*). This then triggers a further conformational change that exposes the fusion domain of the viral transmembrane protein gp41 and enables fusion of the viral and cellular membranes (*4*).

[1]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. [2]Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan. [3]Host and Defense, PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan. [4]Department of Immunology and Molecular Pathology, Windeyer Institute of Medical Sciences, University College London, London W1T 4JF, UK. [5]Retrovirus Research Laboratory, Institute of Comparative Medicine, University of Glasgow, Glasgow G61 1QH, UK.

*These authors contributed equally to this work
†To whom correspondence should be addressed. E-mail: b.willett@vet.gla.ac.uk

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human   1   ..MCVGARRLGRGPCAALLLLGLGLSTVTGLHCVGDTYPSNDRCCHECRP
feline  1   MRVVVGAQRP.RAPHSAVQLLGLVLGTAAALHCVGNTYPKDGKCCSECPP

human   49  GNGMVSRCSRSQNTVCRPCGPGFYNDVVSSKPCKPCTWCNLRSGSERKQL
feline  50  GYGMESRCSGDQDTKCLQCASGFYNEAVNYEPCKPCTQCNQRSGSEPKQR

human   99  CTATQDTVCRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQACKPWTNCT
feline  100 CTPTQDTVCRCRPGTEPQDGYDRGVDCAPCPPGHFSPGDDQACKPWTNCT

human   149 LAGKHTLQPASNSSDAICEDRDPPATQPQETQGPPARPITVQPTEAWPRT
feline  150 LAGKRTLRPASQGSDAVCEDRSPPATTPWETQGPPVRPPTTQPTTAWPRT

human   199 SQGPSTRPVEVPGGRAVAAILGLGLVLGLLGPLAILLALYLLRRDQRLPP
feline  200 SQEPFTPPAEPPRGPQLAAVLGLG..LGLLAPVAAALALLLHHRAWRLPP
                         TM
human   249 DAHKPPGGGSFRTPIQEEQADAHSTLAKI 277
feline  248 ......GGNSFRTPIQEEHADANSTLAKI 270
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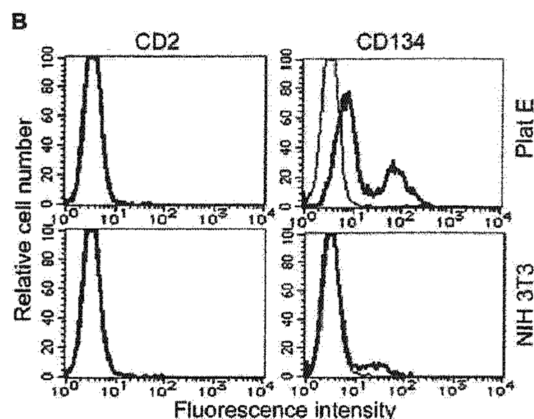

Fig. 1. Molecular cloning and characterization of feline CD134. (A) Alignment of feline and human CD134. Homologous amino acids are shaded light gray; identical residues are shaded dark gray. TM, transmembrane region. (B) Binding of FIV to CD134-expressing cells. Cell cultures transduced with CD134 or CD2 were incubated with culture supernatant from FIV-infected (thick line) or uninfected (thin line) cells, followed by FIV Env detection by flow cytometry.

The feline immunodeficiency virus (FIV) is unique among the nonprimate lentiviruses in that in its natural host species, the domestic cat, it induces a disease similar to AIDS, characterized by a progressive depletion of CD4$^+$ T lymphocytes (*5*). Consequently, an immunodeficiency syndrome develops that is characterized by wasting, neurological manifestations, chronic stomatitis and gingivitis, and an increased incidence of lymphoma (*6*). However, CD4 is not the primary receptor for FIV, suggesting that the virus may bind to an alternative molecule in order to target CD4$^+$ T cells (*7*). Partial elucidation of the mechanism of infection with FIV was provided by the discovery that primary isolates and laboratory strains of virus use CXCR4 as a cofactor for infection (*8*, *9*). However CXCR4 expression alone was insufficient to confer susceptibility to infection with primary isolates of FIV, indicating the existence of an as yet unidentified primary receptor for the virus.

To identify the primary receptor for FIV, we generated a cDNA library in the pMX retroviral vector from an interleukin-2 (IL-2)–dependent CD4$^+$ feline T cell line (MYA-1), which is highly susceptible to infection with FIV (*10*). After transduction of P3U1 murine myeloma cells with the library, cells capable of binding FIV were identified by "panning" with FIV-coated plates (*11*). Positive colonies were expanded, genomic DNA was prepared, and the cDNA insert was amplified from the retroviral vector by polymerase chain reaction. Of three cDNAs cloned from the library, only one showed reproducible binding to FIV when reexpressed in P3U1 cells and was characterized as the feline homolog of CD134 (DNA Data Bank of Japan accession no. AB128982). CD134 was first described as MRC OX-40, an antigen expressed on activated rat CD4$^+$ T lymphocytes and a member of the tumor necrosis factor–nerve growth factor receptor family (*12*, *13*). The feline CD134 cDNA identified here predicts a 270–amino acid protein with strong identity to human CD134 (Fig. 1A). To confirm that the feline CD134 protein binds FIV, we expressed the cDNA in the human cell line Plat-E and the murine cell line NIH-3T3. Specific FIV binding was detected on Plat-E and NIH 3T3 cells transduced with CD134 but not with cells expressing CD2 from the same vector (Fig. 1B).

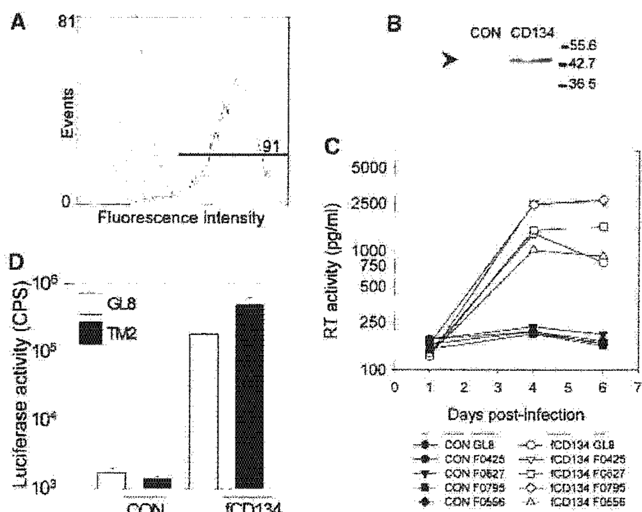

Fig. 2. CD134 expression renders feline cells permissive to infection with FIV. (A) Flow cytometric analysis of CD134 expression on MCC cells. Control antibody (solid histogram) and anti-CD134 (Ber-ACT35, open histogram) are shown. The percentage positive is shown. (B) Immunoblot analysis with rabbit antibody to rat OX40. MCC cells transduced with vector only (CON) were compared with cells transduced with the CD134 expression vector. A ~45-kD species was detected in CD134-transduced cells (arrowhead). (C) Feline (f) CD134 expression renders MCC cells permissive of productive infection with primary strains of FIV. MCC-CON or MCC-CD134 cells were infected with the GL8, F0425, F0827, F0795, and F0556 primary isolates of FIV. Reverse transcriptase activity in the culture supernatants was measured at 1, 4, and 6 days after infection. (D) Luciferase activity in MCC-CON or MCC-CD134 cells infected with HIV (FIV) luciferase pseudotypes bearing the GL8 or TM2 Envs (mean ± SE, n = 4 replicates).

To assess whether CD134 acts as a functional viral receptor, the feline large granular lymphoma cell line MCC (these cells are refractory to infection with primary strains of FIV) was stably transduced with a retroviral vector bearing the CD134 cDNA. Surface CD134 expression was detected by flow cytometry (Fig. 2A), whereas a ~45-kD species was detected by immunoblotting (Fig. 2B). CD134 expression rendered MCC cells permissive for infection with the five primary strains of FIV tested (Fig. 2C), whereas cells transduced with vector only remained nonpermissive. To quantify viral entry into the CD134-expressing cells, we prepared HIV pseudotypes bearing FIV Env proteins and carrying a luciferase gene. Each successful entry event would trigger luciferase production in the target cells. MCC-CD134 cells were permissive for infection with pseudotypes bearing the Env proteins of either the GL8 or TM2 strains of FIV (Fig. 2D),

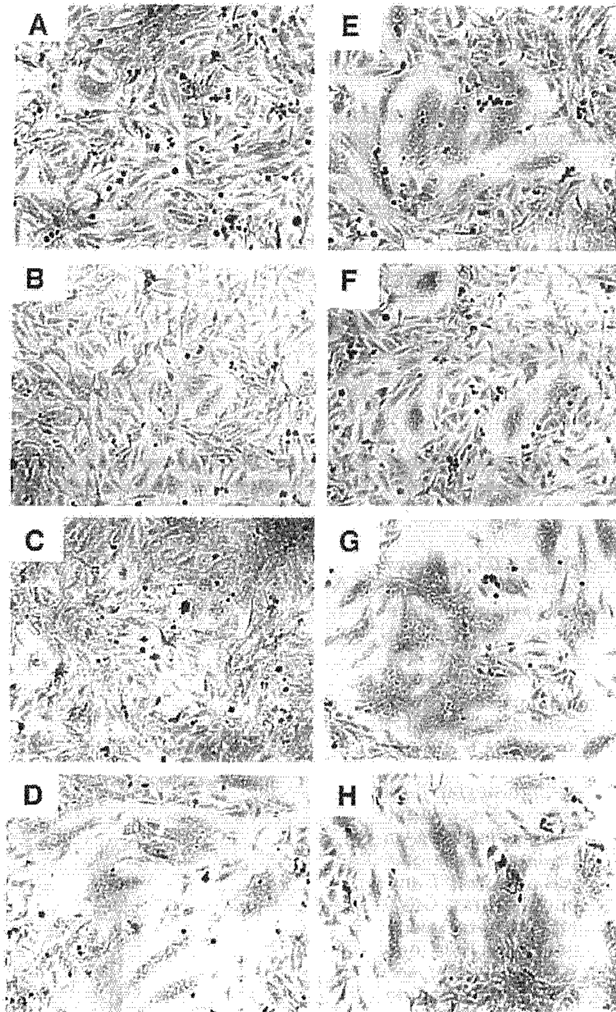

Fig. 3. CD134 expression renders AH927 cells permissive for syncytia formation mediated by FIV Env. FeLV-CR–expressing cells (**A** to **D**) or CD134-expressing cells (**E** to **H**) were transfected with pVR1012 expressing the GL8 [(A) and (E)], F0425 [(B) and (F)], F0827 [(C) and (G)], or PET [(D) and (H)] *env* genes.

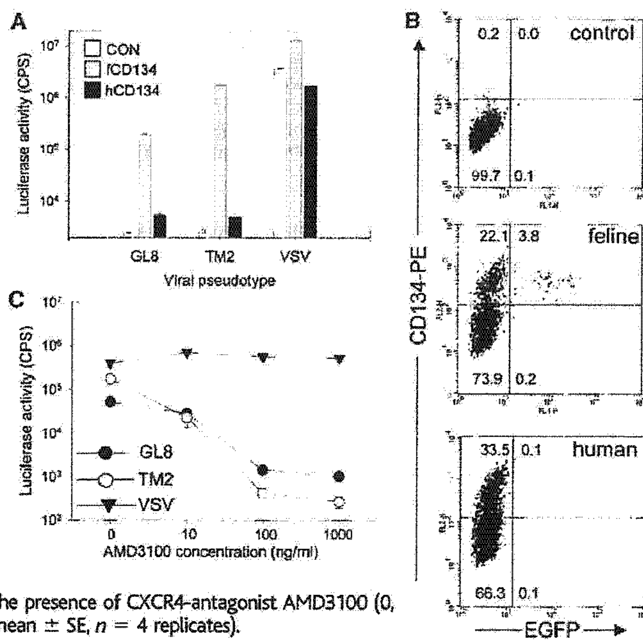

Fig. 4. CD134-mediated infection is species specific and CXCR4-dependent. (**A**) HeLa cells transduced with retroviral vectors bearing feline or human (h) CD134 cDNAs or a vector-only control were infected with HIV (FIV) (GL8 or TM2) or HIV (VSV) luciferase pseudotypes (mean ± SE, $n = 4$ replicates). (**B**) CD134-dependent infection of HeLa cells expressing feline CD134 by HIV (FIV) pseudotypes bearing a GFP marker gene. CD134 and GFP expression were analyzed by flow cytometry. (**C**) HeLa cells expressing feline CD134 were infected with HIV (FIV) (GL8 or TM2) or HIV (VSV) luciferase pseudotypes in the presence of CXCR4-antagonist AMD3100 (0, 10, 100, or 1000 ng/ml) (mean ± SE, $n = 4$ replicates).

confirming that CD134 expression overcame the block to viral entry into these cells. CD134 expression also relieved the block to infection in the feline fibroblast line AH927, whereas a control vector encoding the feline leukemia virus (FeLV)-C receptor (FeLV-CR) had no effect (fig. S1).

Syncytium formation occurs when Env expressed on the surface of a cell comes into contact with an adjacent cell expressing the viral receptor and coreceptor (*4*). Thus, transfection of cells expressing the viral receptor and coreceptor with *env* expression vectors should trigger syncytium formation. Accordingly, transfection of AH927-CD134 cells with constructs bearing the *env* genes from primary isolates (Fig. 3, E to G) led to the development of syncytia, whereas no syncytia were observed in the control cultures (Fig. 3, A to C) expressing the FeLV-CR. In contrast, FIV-PET *env* [derived from a FIV strain analogous to CD4-independent strains of HIV (*14*)] induced syncytium formation in both the CD134 (Fig. 3H) and FeLV-CR–expressing (Fig. 3D) cells, consistent with this Env interacting with CXCR4 in the absence of a primary receptor (*15*).

Human cells are refractory to infection with primary strains of FIV (*16*). We therefore examined the species specificity of the interaction between FIV and CD134. The human cell line HeLa was stably transduced with either feline or human CD134, and surface expression of CD134 was confirmed by flow cytometry (Fig. 4B). Although ectopic expression of feline CD134 rendered HeLa cells permissive for infection with primary FIV strain GL8 or TM2 pseudotypes (Fig. 4A), human CD134 expression had little effect. In contrast, the susceptibility of the feline and human CD134-expressing cells to HIV pseudotypes with a vesicular stomatitis virus glycoprotein (VSVG) envelope was broadly similar to that of the control HeLa cells. Similarly, feline, but not human, CD134 expression permitted infection with HIV pseudotypes bearing FIV envelopes and encoding green fluorescent protein (GFP) (Fig. 4B). These results suggest that the specificity of the interaction between the virus and its primary receptor may be a major determinant of the species specificity of FIV.

Because infection with both primary and cell culture–adapted strains of FIV is CXCR4-dependent (*8*, *9*), we determined whether CD134-dependent infection required coexpression of CXCR4. HeLa cells express high levels of CXCR4, and human CXCR4 is an efficient coreceptor for FIV (*17*). Infection of HeLa-CD134 cells with GL8 and TM2 pseudotypes was inhibited efficiently by the selective CXCR4 antagonist AMD3100, whereas infection with control HIV (VSV) pseudotypes was not affected (Fig. 4C), confirming an absolute requirement for both receptor and coreceptor.

Further evidence for a role for CD134 in viral infection was provided by down-regulation of CD134 from the surface of FIV-infected cells (fig. S2). Moreover, CD134-expressing cells expressed levels of CXCR4 similar to those expressed by control cells, indicating that CD134 did not mediate its effect on FIV infection by modulating CXCR4 expression (fig. S3).

We have shown that CD134 functions as a primary receptor for an immunodeficiency-causing lentivirus. CD134 expression is largely restricted to $CD4^+$ T lymphocytes (*12*, *18*–*20*); however, in humans and mice, CD134 is also expressed at lower levels on activated $CD8^+$ T cells (*18*, *21*), macrophages, and activated B cells (*22*). $CD4^+$ T cells are the primary target for FIV in early infection, whereas in chronic infection $CD8^+$ T cells and B cells are also infected (*23*, *24*). The tropism of FIV in vivo therefore appears to be consistent with the predicted expression of CD134. In addition, the viral coreceptor CXCR4 is expressed widely in the cat [in activated T cells, B cells, and monocytes (*25*)], and because some primary and cell culture–adapted strains of FIV can infect via CXCR4 alone (*26*) (CD134-independent infection), the broadening of cell tropism of the virus in chronic infection may represent a shift toward CD134-independent infection.

Signaling through CD134 plays a crucial role in the survival and proliferation of $CD4^+$ T cells that have encountered antigen (*20*). By targeting CD134-expressing cells, FIV would selectively deplete a subset of $CD4^+$ T cells that is integral to the development of antigen-specific T cell responses. In contrast, by using CD4 as a primary receptor, HIV has the potential to infect all $CD4^+$ T cells and induce a more profound immune defect. However, the cell tropism of HIV is restricted by the expression of the viral coreceptor which, for the majority of strains that are transmitted, is CCR5. CCR5 expression on $CD4^+$ T cells is restricted to an effector/memory T cell subset (*27*, *28*). Thus, despite the use of distinct primary binding receptors, both the human and feline viruses selectively impair antigen-specific helper T cell responses.

Vaccination may lead to enhancement of infection in the feline model of AIDS (*29*). Our new data may shed new light on the mechanism of enhancement, because CD134 is a T cell activation antigen, with expression in vivo restricted predominately to $CD4^+$ T cells. Vaccination may induce an expansion of a population of cells expressing the viral receptor, so that if sterilizing immunity is not achieved, vaccination may prove counterproductive.

That two lentiviruses with host species as divergent as human beings and the domestic cat should use distinct primary receptors to target similar T cell subsets underlines the central role of $CD4^+$ T lymphocyte infection in the pathogenesis of AIDS. Whether the feline and human lentiviruses evolved from a common ancestor, such as a CD4- or CD134-independent virus, is an intriguing question regarding the development of viral virulence, and this study represents a first step toward providing a solution.

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Materials and Methods
SOM Text
Figs. S1 to S3
References

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Regulation of Fasted Blood Glucose by Resistin

Ronadip R. Banerjee,[1]* Shamina M. Rangwala,[1]* Jennifer S. Shapiro,[1] A. Sophie Rich,[1] Ben Rhoades,[1] Yong Qi,[1] Juan Wang,[1] Michael W. Rajala,[2] Alessandro Pocai,[2] Phillipp E. Scherer,[2] Claire M. Steppan,[1] Rexford S. Ahima,[1] Silvana Obici,[2] Luciano Rossetti,[2] Mitchell A. Lazar[1]†

The association between obesity and diabetes supports an endocrine role for the adipocyte in maintaining glucose homeostasis. Here we report that mice lacking the adipocyte hormone resistin exhibit low blood glucose levels after fasting, due to reduced hepatic glucose production. This is partly mediated by activation of adenosine monophosphate–activated protein kinase and decreased expression of gluconeogenic enzymes in the liver. The data thus support a physiological function for resistin in the maintenance of blood glucose during fasting. Remarkably, lack of resistin diminishes the increase in post-fast blood glucose normally associated with increased weight, suggesting a role for resistin in mediating hyperglycemia associated with obesity.

The parallel epidemics of obesity and type 2 diabetes suggest a relation between increased adipose mass and insulin resistance (*1*). Adipocytes secrete several signaling molecules that affect glucose homeostasis, such as fatty acids, adiponectin, leptin, interleukin-6, and tumor necrosis factor–α (*2*). Resistin is an adipocyte-secreted hormone that has been linked to diabetes (*3*, *4*), a view supported by increased blood glucose and increased hepatic glucose production when resistin is administered acutely in rodents (*5*, *6*). However, the role of resistin in glucose metabolism is controversial (*7*), and the normal physiological function of resistin is unknown.

[1]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, and The Penn Diabetes Center, 611 CRB, 415 Curie Boulevard, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA. [2]Department of Medicine, Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed: E-mail: lazar@mail.med.upenn.edu

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Short Communication

Downmodulation of CD3ε expression in CD8$\alpha^+\beta^-$ T cells of feline immunodeficiency virus-infected cats

Yorihiro Nishimura,[1]† Masayuki Shimojima,[1]‡ Eiji Sato,[1]§ Yoshihiro Izumiya,[1]|| Yukinobu Tohya,[1] Takeshi Mikami[1]¶ and Takayuki Miyazawa[1,2,3]

Correspondence
Takayuki Miyazawa
takavet@obihiro.ac.jp

[1]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

[2]Host and Defense, PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

[3]Laboratory of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

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Feline immunodeficiency virus (FIV) infection in cats is associated with an increase of feline CD (fCD)8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells in peripheral blood. To investigate these cells in more detail, an anti-fCD3ε mAb, termed NZM1, was generated, which recognizes the extracellular epitope of the fCD3ε molecule. The anti-fCD3ε mAb proved to be more suitable for identifying feline T cells than the anti-fCD5 one, which has been used as a pan-T-cell reagent in cats, because of the presence of fCD5$^+$fCD3ε^- cells among lymphocytes. Although the fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells in the FIV-infected cats expressed fCD3ε, a subset of fCD8$\alpha^+\beta^-$ cells expressed fCD3ε antigen at a lower level than the T cells whose phenotype was fCD4$^+$, or fCD8$\alpha^+\beta^{low}$. The lower expression of fCD3ε may be associated with the immune status of fCD8$\alpha^+\beta^-$ T cells.

CD8$^+$ T cells play potential roles in the immunopathogenesis of human immunodeficiency virus type 1 (HIV-1) infection (Yang & Walker, 1997). The CD8 antigen consists of two polypeptides, CD8α and CD8β, and exists as a heterodimer (CD8$\alpha\beta$) or a homodimer (CD8$\alpha\alpha$). In humans, T-cell receptor (TCR) $\alpha\beta$ T cells express the CD8$\alpha\beta$ heterodimer, and TCR$\gamma\delta$ T cells and natural killer cells express the CD8$\alpha\alpha$ homodimer (Moebius *et al.*, 1991). Feline immunodeficiency virus (FIV) infection in cats has been studied extensively as an animal model for the persistent infections and pathogenesis caused by HIV (for a review see Miyazawa, 2002). Previously, we found that feline CD (fCD)8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells increased in number in the peripheral blood of FIV-infected cats (Shimojima *et al.*, 1998a). These subsets were reported to play roles in the suppression of FIV replication (Bucci *et al.*, 1998; Flynn *et al.*, 2002; Gebhard *et al.*, 1999; Shimojima *et al.*, 2004). The induction of similar subpopulations was also confirmed in human diseases, such as HIV infection (Schmitz *et al.*, 1998). However, it remains unknown whether the fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells are T cells or natural killer cells. The phenotypic characterization of fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells in FIV-infected cats is difficult due to a lack of monoclonal antibodies (mAbs) against appropriate surface markers.

Cells of the T-cell lineage bear a TCR–CD3 complex consisting of variable $\alpha\beta$ or $\gamma\delta$ TCR chains associated with invariant CD3 chains of γ, δ, ε and ζ (Ashwell & Klausner, 1990). The CD3ε chain appears to be the most immunogenic and exposed part of CD3, as anti-human CD3 mAbs are predominantly directed to epitopes of the CD3ε subunit (Transy *et al.*, 1989). Only completely assembled TCR–CD3 complex can be expressed on the T-cell surface (Clevers *et al.*, 1988). Therefore, mAbs for CD3ε have exquisite specificity for T cells and are widely used to identify T cells

†Present address: Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama-shi, Tokyo 208-0011, Japan.

‡Present address: Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108-8639, Japan.

§Present address: Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA.

||Present address: Department of Biological Chemistry, UC Davis School of Medicine, UC Davis Cancer Center, Sacramento, CA 95817, USA.

¶Present address: Food Safety Commission, The Cabinet Office, Japanese Government, Nagata-cho, Chiyoda-ku, Tokyo, Japan.

in both humans (Reinherz *et al.*, 1979) and mice (Leo *et al.*, 1987). To investigate feline T cells, Joling *et al.* (1996) reported that an anti-human CD3ε polyclonal antibody, prepared from rabbits immunized with peptides of the cytoplasmic domain of human CD3ε, cross-reacted with feline CD3ε and could be used for immunohistochemical studies in cats. However, this antibody was inconvenient as the permeabilization of cells is necessary for flow cytometric analysis. Instead of a specific anti-fCD3 mAb, f43 mAb, which recognizes the feline homologue of the CD5 antigen, has been used as a pan-T-cell reagent in cats (Ackley & Cooper, 1992). However, the CD5 molecule is also expressed on a subset of B cells in humans, rabbits and mice (Caligaris-Cappio *et al.*, 1982; Manohar *et al.*, 1982; Raman & Knight, 1992), therefore f43 mAb appears to be inappropriate for the detection of feline T cells. In order to solve this problem, we prepared a mAb termed NZM1 that detects the fCD3ε antigen in immunoblotting and flow cytometric analyses, and characterized the fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells in FIV-infected cats.

Hybridomas were generated from BALB/c mice immunized with insect cells (*Sf*9 cells) infected with the recombinant baculovirus rAcfCD3ε, which carries cDNA encoding the fCD3ε molecule (Nishimura *et al.*, 1998). A positive hybridoma designated NZM1 (IgG3) was selected based on the reactivity with a T-lymphoblastoid cell line, MYA-1 cells (Miyazawa *et al.*, 1989b), by an indirect immunofluorescence assay using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The specificity of NZM1 was confirmed by the immunoblotting analysis using *Sf*9 cells infected with rAcfCD3ε and feline peripheral blood mononuclear cells (PBMCs) as antigens (Fig. 1). As a control, a rabbit polyclonal antibody against the cytoplasmic region of human CD3ε (Dako A/S) was used. Secondary antibodies conjugated with horseradish peroxidase were used to detect positive signals as described previously (Miyazawa *et al.*, 1989a). NZM1 recognized several bands of about 25 kDa in *Sf*9 cells infected with rAcfCD3ε (Fig. 1, lane 8) but not in mock-infected cells (Fig. 1, lane 6) or cells infected with the control baculovirus (Fig. 1, lane 7). NZM1 was confirmed to react with a 25 kDa molecule of MYA-1 cells (Fig. 1, lane 9) and feline PBMCs (Fig. 1, lane 10), which was identical to the molecule recognized by the anti-human CD3ε polyclonal antibody (Fig. 1, lanes 1–5). These findings indicate that the mAb NZM1 is directed against the fCD3ε molecule.

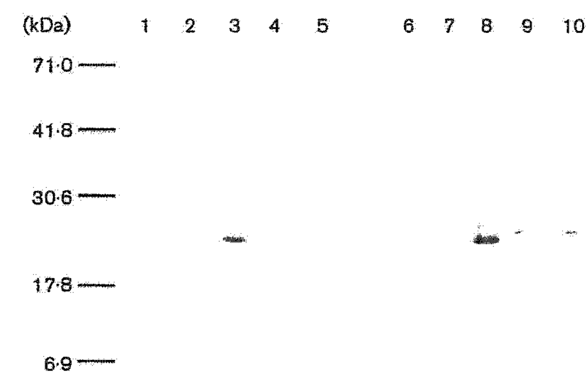

Fig. 1. Immunoblotting analysis of *Sf*9 cells (lanes 1–3 and 6–8), MYA-1 cells (lanes 4 and 9) and feline PBMCs (lanes 5 and 10) using anti-human CD3ε polyclonal antibody (lanes 1–5) and NZM1 mAb (lanes 6–10). Positive reactions were visualized by 3,3′-diaminobenzidine tetrahydrochloride staining. The *Sf*9 cells were mock-infected (lanes 1 and 6) or infected with the control baculovirus (lanes 2 and 7) or rAcfCD3ε (lanes 3 and 8). Specific bands were observed in lanes 3–5 and 8–10.

Next, we investigated whether the engagement of fCD3ε with NZM1 also induced T-cell proliferation as demonstrated with anti-CD3 mAbs of other species (Leo *et al.*, 1987; Tsoukas *et al.*, 1985; Yang *et al.*, 1996). Feline PBMCs (2×10^5) separated from heparinized whole blood of a specific-pathogen-free (SPF) cat were suspended in 100 μl RPMI 1640 medium containing fetal calf serum (10 %, v/v) and antibiotics, and plated in a well of a 96-well flat-bottomed microculture plate. The PBMCs were cultured in the presence of the anti-fCD4 mAb [4D9 (IgG1); Shimojima *et al.*, 1997], anti-fCD8α [12A3 (IgG2a); Shimojima *et al.*, 1998b] or NZM1 (final dilution, ascites $1:10^3$, $1:10^4$ or $1:10^5$) for 72 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. The proliferation of PBMCs was measured by MTT assay (Mosmann, 1983). The cells proliferated to a greater extent when cultured with NZM1 than with 4D9 or 12A3 ($P<0\cdot005$, $n=3$; data not shown). We considered that NZM1 recognizes the extracellular epitope of fCD3ε, as it could stain feline PBMCs without permeabilization in the immunofluorescence analysis and induce the proliferation of feline PBMCs in the co-cultivation experiments.

Two cats infected with each of the FIV TM1 (cat 103) and TM2 (cat 104) strains for 11 years (Miyazawa *et al.*, 1989a) and one infected with the Petaluma strain for 2 years (cat 115) were used in the flow cytometric analysis. Three adult SPF cats aged 8–10 years (cats 102, 201 and 202) were used as uninfected controls. All cats were clinically healthy. PBMCs were suspended in a sorter buffer (PBS containing 3 % fetal calf serum and 0·05 % sodium azide) and centrifuged at 800 r.p.m. to remove platelets. The mAb NZM1 was labelled with FITC (fCD3ε–FITC) according to a standard procedure. PBMCs were washed twice in the cold sorter buffer and incubated with fCD3ε–FITC. After washing with the sorter buffer, stained cells were analysed after gating for lymphocytes based on light (forward and side) scatters using a flow cytometer FACScan with CELLQUEST software (Becton Dickinson). The different subpopulations were expressed as percentages of the total lymphocyte population. The uninfected and FIV-infected groups gave distinctive patterns of fCD3ε expression, and representative results are shown in Fig. 2. In FIV-uninfected SPF cats, the fCD3ε molecule was expressed on $57\cdot2\pm9\cdot5$ % ($n=3$) of

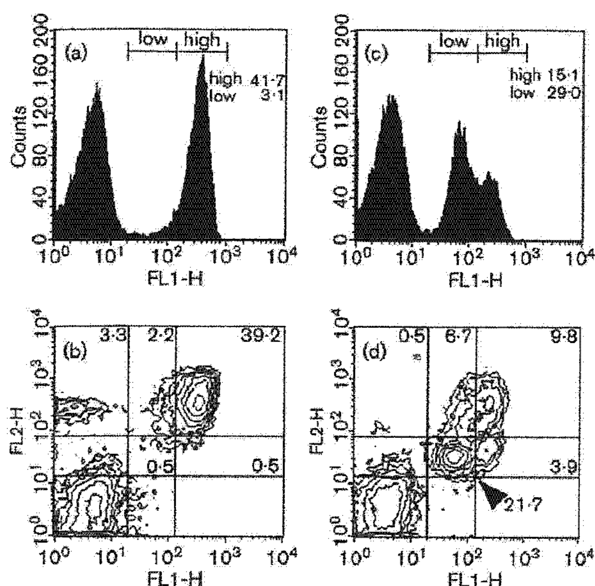

Fig. 2. Flow cytometric analysis of feline peripheral blood lymphocytes. Isolated PBMCs were stained with fCD3ε–FITC (NZM1, FL1-H) only (a, c) or fCD3ε–FITC and fCD5–PE (f43, FL2-H) (b, d). The *x* axis gives the fluorescent intensity for fCD3ε. The *y* axis shows the fluorescent intensity for fCD5 (b, d). Numbers in the corner of each panel indicate the percentage of cells expressing fCD3ε at indicated levels (a, c) or the percentage of cells in each area (b, d). Three cats infected with FIV and three SPF cats as uninfected controls were used. Representative results of uninfected (cat 102) (a, b) and FIV-infected (cat 104) (c, d) cats are shown.

peripheral lymphocytes (Fig. 2a). On the other hand, two subsets of fCD3$^+$ cells, fCD3^{high} (33·1±16·5 %, $n=3$) and fCD3^{low} (20·7±9·3 %, $n=3$), were detected in the FIV-infected cats (Fig. 2c). As the fCD5 antigen has been considered a pan-T-cell molecule in cats, PBMCs were labelled with fCD3ε–FITC and phycoerythrin (PE)-conjugated anti-fCD5 mAb (fCD5–PE), f43 (Ackley & Cooper, 1992) and analysed by flow cytometry (Fig. 2b, d). Although most of the fCD5 cells expressed the fCD3ε molecule, there was a substantial number of fCD5$^+$fCD3ε$^-$ cells in FIV-uninfected SPF cats (2·0±1·7 %, $n=3$; Fig. 2b). So anti-fCD5 mAb appears to be unsuitable for the detection of feline T cells. The expression of fCD5 antigen on feline B cells has not been characterized in detail, and it is unknown whether this subset corresponds to CD5$^+$ B cells in humans and mice. It should also be noted that the fCD5^{low} population consisted of fCD3ε^{high} and fCD3ε^{low} subsets (Fig. 2d), which indicates that fCD8$\alpha^+\beta^-$ cells in FIV-infected cats consist of fCD3ε^{high} and fCD3ε^{low} subsets (Shimojima *et al.*, 1998a; Stievano *et al.*, 2003).

Next the PBMCs were stained with mAb fCD3ε–FITC and either fCD4–PE (Fel7; Ackley *et al.*, 1990), fCD8β–PE (FT2; Klotz & Cooper, 1986), fCD8α (2D7; Shimojima *et al.*, 1998b), or the mixture of fCD4–PE, fCD8α and fCD8β–PE. A secondary rat anti-mouse IgG2a antibody conjugated with PE (Zymed Laboratories) was used for the detection of fCD8α. The uninfected and FIV-infected groups gave distinctive patterns, and representative results are shown in Fig. 3. Most of the fCD4$^+$ and fCD8$^+$ cells were fCD3ε$^+$. The fCD3ε$^+$ cell population consisted of fCD4$^+$ (46·3±2·4%; Fig. 3a), fCD8α$^+$ (41·9±2·3 %; Fig. 3b) and fCD4$^-$ fCD8$\alpha^-\beta^-$ (9·3±0·6 %; Fig. 3d) cells in the SPF cats ($n=3$). Most of the fCD3^{low} cells in the FIV-infected cats were fCD5^{low} fCD4$^-$ fCD8$\alpha^{low}\beta^-$ (Fig. 3d–g). In addition, fCD8β^{low} cells whose population expanded in FIV-infected cats also expressed fCD3ε (Fig. 3c, g).

The fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells in the FIV-infected cats expressed fCD3ε, hence these subsets are T cells. It is still unknown at present whether fCD8$\alpha^+\beta^-$, fCD8$\alpha^+\beta^{low}$ and fCD3ε$^+$ fCD4$^-$ fCD8$\alpha^-\beta^-$ cells are $\gamma\delta$ T cells, as no reagent specific for the feline TCR γ- or δ-chain is available. We also found a lower level of expression of the fCD8α molecule in fCD8$\alpha^+\beta^-$ subsets. A decreased expression of CD8α is reported in CD3$^+$ cells but not natural killer cells in HIV-infected individuals (Ginaldi *et al.*, 1997). Downregulation of fCD8 expression may contribute to the progressive reduction of fCD8$^+$ cell function in FIV-infected cats. Several factors may be involved in the change of fCD3ε expression in FIV infection. In general, the CD3^{low} T cell is a recently antigen-activated or memory cell. It is reported that both activated and non-activated T cells from HIV-positive patients express less CD3 than those from control subjects (Ginaldi *et al.*, 1997). As CD3ε plays an important role in signalling of TCR/CD3, fCD3ε^{low} cells might raise the activation threshold and contribute to the lack of effective immune surveillance. There is a continuous loss of naïve CD4 and CD8 T cells and expansion of memory cells in HIV-infected patients (Bass *et al.*, 1992). As the majority of fCD8$\alpha^+\beta^-$ cells show an increase in fCD11a expression, one of the activation antigens (Shimojima *et al.*, 2003) and CD8$\alpha^+\beta^-$ memory T cells descend directly from clonally expanded CD8$\alpha^+\beta^+$ T cells (Konno *et al.*, 2002), we speculate that fCD3ε^{low}fCD8$\alpha^+\beta^-$ T cells consist of activated memory subsets. Hohdatsu *et al.* (2003) reported controversial anti-FIV activities of fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ subsets. Not all fCD8$\alpha^+\beta^-$ and fCD8$\alpha^+\beta^{low}$ cells, but some with enough fCD3ε expression, may have strong anti-FIV activity.

Trimble & Lieberman (1998) reported the expansion of CD3ζ$^-$ subsets in a substantial fraction of CD8$^+$ T cells in HIV-infected patients. They classified the CD8$^+$ cells into the subpopulations CD8$^+$CD3ζ$^-$ and CD8$^+$CD3ζ$^+$. They did not mention the fluorescent intensity of the CD3ε molecule on CD3$^+$ cells, and concluded that the downregulation of CD3ε expression is independent of other TCR/CD3 components. A decrease in CD3ζ mRNA levels was also reported in T cells from AIDS patients (Geertsma *et al.*, 1999), but that of CD3ε mRNA levels has not yet been discussed. Although downregulation of CD3

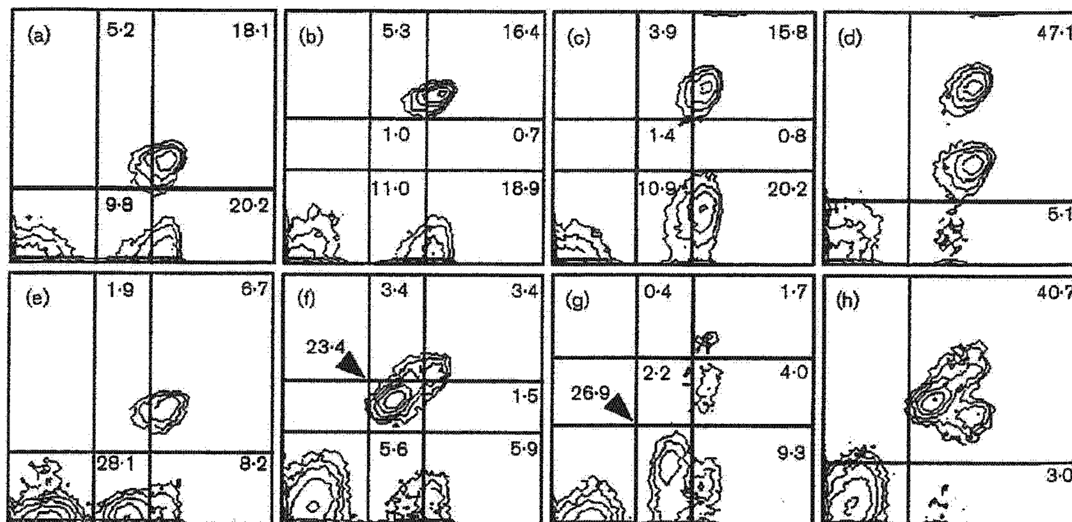

Fig. 3. Two-colour flow cytometric analysis of feline peripheral blood lymphocytes. Isolated PBMCs were stained with fCD3ε–FITC (NZM1) and either fCD4–PE (Fel7) (a, e), fCD8α (2D7) (b, f), fCD8β–PE (FT2) (c, g) or a mixture of fCD4–PE, fCD8α and fCD8β–PE (d, h). Binding of fCD8α was visualized using PE-conjugated secondary antibody. The *x* and *y* axes show fluorescent intensities for fCD3ε and molecules, respectively. Numbers in the corner of each panel indicate the percentage of cells in each area. Three cats infected with FIV and three SPF cats as uninfected controls were used. Representative results for uninfected (cat 202) (a–d) and FIV-infected (cat 104) (e–h) cats are shown.

expression on $CD4^+$ and $CD8^+$ cells is reported in HIV-infected patients, its relationship with CD3ζ expression is unclear (Ginaldi *et al.*, 1997). In the fCD3 complex, fCD3ε is the only molecule whose cDNA has been identified, and NZM1 is the first mAb specific to the fCD3 component. Therefore it is not known at present whether the fCD3ε downregulation involves a decrease of other feline TCR/CD3 components, including fCD3ζ. If the downregulation of fCD3ε in the $fCD8^+$ cells of FIV-infected cats correlates with disease progression, as does that of CD3ζ in HIV infection (Geertsma *et al.*, 1999), the measurement of fCD3ε expression may contribute to our understanding of the immune status of FIV-infected cats.

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A novel antigenic variant of *Canine parvovirus* from a Vietnamese dog

Brief Report

M. Nakamura[1], Y. Tohya[1], T. Miyazawa[2,3], M. Mochizuki[4], H. T. T. Phung[1], N. H. Nguyen[5], L. M. T. Huynh[5], L. T. Nguyen[5], P. N. Nguyen[6], P. V. Nguyen[6], N. P. T. Nguyen[6], and H. Akashi[1]

[1]Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[2]Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan
[3]Host and Defense, PRESTO, Japan Science and Technology Agency, Kawaguchi, Japan
[4]Laboratory of Clinical Microbiology, Kyoritsu Seiyaku Corporation, Tokyo, Japan
[5]Department of Veterinary Microbiology, Infectious Diseases and Pathology, Faculty of Veterinary and Animal Science, Hanoi Agricultural University, Hanoi, Vietnam
[6]Department of Parasitology, Microbiology and Pathology, Faculty of Animal Science and Veterinary Medicine, University of Agriculture and Forestry, Ho Chi Minh City, Vietnam

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Summary. Nine isolates of *Canine parvovirus* (CPV) were obtained from Vietnamese dogs and cats. One canine isolate showed a unique antigenic property which indicates a novel antigenic variant of CPV-2b when examined with hemagglutination inhibition tests using our monoclonal antibodies, 21C3 and 19D7, which were recently developed. This isolate had an amino acid substitution of residue 426, Asp to Glu, and the same substitution has recently been found in CPV from Italian dogs. This study first showed that such substitution caused an antigenic difference demonstrable by monoclonal antibodies and that a similar evolution may have occurred in CPV in Vietnam.

*

Canine parvovirus (CPV) is a small, non-enveloped virus that possesses single-stranded DNA. The CPV capsid is composed of two structural proteins, VP1 and

VP2, which are translated from alternatively spliced mRNAs [20]. VP2 is a main component of capsid and amino acid substitutions in VP2 cause antigenic changes of CPV [18, 21].

As CPV has shown several antigenic and host range changes since its emergence, it is thought to be an interesting model of viral evolution. CPV or CPV-2 suddenly emerged in dogs in the late 1970's and rapidly spread worldwide [6]. After the emergence of CPV-2, two new antigenic variants, designated CPV-2a and CPV-2b, have arisen consecutively [17, 19]. These two variants have almost completely replaced CPV-2 and have been distributed worldwide [7, 8, 17, 22]. Five conserved amino acid differences in VP2 are observed between CPV-2 and CPV-2a [19]. CPV-2b has two additional substitutions in VP2 of residue 426 Asn (Asn-426) to Asp and Ile-555 to Val [19]. Asp-426 is an important substitution that distinguishes CPV-2b from the other antigenic types, including the related feline panleukopenia virus (FPLV) and mink enteritis virus (MEV) [19].

Recently, another antigenic change was observed. We isolated CPV-2a- and CPV-2b-related viruses from domestic and leopard cats in Vietnam [10, 11, 13]. Three isolates from leopard cats were shown to be a new antigenic type by the absence of reactivity with several monoclonal antibodies (MAbs) [11]. They were designated CPV-2c and further divided into CPV-2c(a) and CPV-2c(b) by variation of residue 426, which distinguishes CPV-2b from CPV-2a. CPV-2c viruses have the substitution of Gly-300 to Asp, which is thought to be responsible for the characteristic antigenicity of them.

The emergence of CPV-2c indicates that CPV is still evolving in Vietnam. Therefore, it is important to research on field isolates in this area. In this study, we isolated CPV from rectal swab samples of dogs and cats collected in Vietnam and determined genetic and antigenic properties of the isolates. Interestingly, one canine isolate showed a unique antigenic property which indicates a novel antigenic variant of CPV-2b.

Eighty-six rectal swab samples from domestic dogs and 40 rectal swab samples from domestic cats were collected in Ho Chi Minh City and Hanoi in Vietnam in 2002. Samples were suspended in Dulbecco's modified Eagle's medium and filtrated through Millipore filter (pore size 0.22 μm). Samples were inoculated onto Crandell feline kidney (CRFK) cells, Madin-Darby canine kidney (MDCK) cells, or the thymic lymphoma cell line 3201, followed by blind passages one to three times until cytopathic effects (CPEs) were observed. The isolates were propagated in CRFK or MDCK cells and used for antigenic and sequence analyses.

Hemagglutination inhibition (HI) tests were performed for antigenic analysis of Vietnamese isolates as previously described [12]. The HI titer was determined as the reciprocal of the highest dilution that completely inhibited viral hemagglutination. MAbs A3B10, B6D5, B4E1, A4E3, C1D1, B4A2 [16], and P2-215 [9] were previously reported elsewhere. MAbs 2G5, 21C3, 19D7, and 20G4 were generated in our previous work [15]. The viruses used as reference strains in the antigenic analysis were FPLV TU-1 [14], MEV-2 M-1 [11], CPV-2 CPV-b

[16] and Cp49 [2], CPV-2a CPV-31 [17] and 97-003 [11], CPV-2b CPV-39 [17] and 97-008 [11], CPV-2c(a) V139 [10], and CPV-2c(b) V203 [10]. In addition to Vietnamese isolates, recent isolates from Japanese dogs and cats [unpublished] were also analyzed.

For sequence analysis, the VP2 gene was amplified by PCR with the primer sets reported previously [11]. After the amplified DNA fragments were purified from the agarose gel, they were used for the sequencing reaction with a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). The samples were resolved on an automated DNA sequencer (model 3100-Avant; Applied Biosystems). The sequences of the VP2 genes of the previously published isolates were obtained from the DDBJ database. Phylogenetic analysis was carried out using GENETYX-MAC ver 12.0 (Genetyx Co., Tokyo, Japan). Pair-wise genetic distances were calculated by using the Jukes-Cantor method. Phylogenetic trees were constructed by using the neighbor-joining method and bootstrap analysis was performed with 1000 trials.

Eight isolates from dogs and one isolate from a cat were obtained (Table 1). HNI-3-4 was isolated and propagated in MDCK cells. HCM-8 and HNI-4-1 were isolated in 3201 cells and propagated in CRFK cells. The other six isolates were obtained and propagated in CRFK cells. Seven of the eight isolates obtained from Vietnamese dogs were classified into CPV-2b type by HI assay (Table 1), suggesting that CPV-2b viruses were predominant in the dog population in this area. CPV-2b viruses were isolated from Vietnamese cats in our previous study [10, 11, 13] and also in this study (HNI-1-18) (Table 1). The isolates from Vietnamese dogs were not phylogenetically separated from those from Vietnamese cats (Fig. 1). These data suggest that transmission of CPV-2b between dogs and cats has occurred in Vietnam.

The sequence analysis of the VP2 gene confirmed the classification of these isolates and revealed some amino acid substitutions. HCM-8 had the substitutions of Pro-13 to Ser and Thr-265 to Lys (Table 2). A similar substitution, Thr-265 to Pro, was observed in Italian isolates [3, 4]. HCM-18 and HNI-2-13 had a common substitution of Phe-267 to Tyr (Table 2). Because residues 265 and 267 are not exposed on the capsid surface [1, 25], substitutions of these residues may not affect antigenicity of the viruses. All isolates have the common substitution of Ser-297 to Ala (Table 2), which has been observed in most recent CPV-2a- and CPV-2b-related isolates in Asia [11], Italy [3–5], and Germany [23].

Usually, CPV-2b has been distinguished from CPV-2a by a single MAb B4A2 [19] which recognizes CPV-2a but not CPV-2b. In addition to the MAb, we recently developed an MAb, 21C3, which distinguishes CPV-2b from CPV-2a [15], recognizing CPV-2b but not CPV-2a. By using these MAbs, we can clearly distinguish CPV-2b from CPV-2a. Both MAbs recognize the antigenic site A [15, 21], which is located on the tip of the threefold spike in the capsid structure [24]. This antigenic site A contains the residue 426, which causes antigenic changes of CPV-2a to CPV-2b by substitution of the residue from Asn to Asp. In this study, one isolate, HNI-4-1, showed a unique antigenic property different from

Table 1. HI reactivity of MAbs against Vietnamese isolates

Isolates	Origin	Reactivity with the following MAbs											Antigenic type
		A3B10	B6D5	B4E1	A4E3	C1D1	B4A2	P2-215	2G5	21C3	19D7	20G4	
HCM-6	Dog	NT[a]	NT	NT	NT	NT	NT	NT	32,000	6,400	128,000	3,200	CPV-2b
HCM-8	Dog	12,800	640	10	128	1,280	<4	<1,000	64,000	6,400	128,000	3,200	CPV-2b
HCM-18	Dog	12,800	640	10	128	1,280	<4	<1,000	32,000	6,400	64,000	1,600	CPV-2b
HCM-23	Dog	12,800	640	10	128	1,280	<4	<1,000	64,000	6,400	128,000	3,200	CPV-2b
HNI-2-13	Dog	NT	NT	NT	NT	NT	NT	NT	32,000	6,400	64,000	1,600	CPV-2b
HNI-3-4	Dog	12,800	640	10	128	1,280	<4	<1,000	64,000	6,400	128,000	3,200	CPV-2b
HNI-3-11	Dog	NT	NT	NT	NT	NT	NT	NT	64,000	12,800	128,000	3,200	CPV-2b
HNI-4-1	Dog	12,800	640	10	64	640	<4	<1,000	32,000	400	8,000	3,200	CPV-2b variant
HNI-1-18	Cat	NT	NT	NT	NT	NT	NT	NT	64,000	12,800	128,000	3,200	CPV-2b
FPLV		6,400	640	1,280	<4	<10	32	16,000	<1,000	<100	32,000	<100	Reference strains[b]
MEV		NT	NT	NT	NT	NT	NT	NT	<1,000	<100	64,000	<100	
CPV-2		12,800	1,280	1,280	64	<10	16	<1,000	<1,000	<100	32,000	<100	
CPV-2a		6,400	1,280	20	128	640	16	<1,000	64,000	<100	32,000	3,200	
CPV-2b		6,400	640	10	64	1,280	<4	<1,000	64,000	3,200	128,000	1,600	
CPV-2c(a)		NT	NT	NT	NT	NT	NT	NT	128,000	<100	32,000	512,000	
CPV-2c(b)		NT	NT	NT	NT	NT	NT	NT	64,000	3,200	128,000	128,000	

[a] *NT* not tested

[b] FPLV TU-1, MEV M-1, CPV-2 CPV-b, Cp49, CPV-2a CPV-31, 97-003, CPV-2b CPV-39, 97-008, CPV-2c(a) V139 and CPV-2c(b) V203 were used as reference strains

Fig. 1. Phylogenetic analysis based on the complete VP2 nucleotide sequences of CPV isolates. Sequences of the VP2 gene of the previously published isolates were obtained from the DDBJ database; CPV-2 CPV-b (Accession number: M38245), CPV-2a CPV-31 (M24000), CPV-15 (M24003), FPV-314 (D78585), Taiwan9 (AB054213), LCPV-T1 (AB054214), V120 (AB054215) and V154 (AB054217), CPV-2b CPV-39 (M74849), CPV-133 (M74852), 97-008 (AB115504), V123 (AB054218), V209 (AB054219), V217 (AB054220) and V204 (AB054221), CPV-2c(a) V139 (AB054222) and V140 (AB054223), CPV-2c(b) V203 (AB054224). The trees were constructed by using the neighbor-joining program and bootstrap values were indicated. The number in parentheses means the year when the strain was isolated. *DD*, domestic dog; *DC*, domestic cat; *LC*, leopard cat; *JPN*, Japan; *VTN*, Vietnam; *TWN*, Taiwan

Table 2. Amino acid variation in VP2 protein

Isolates	Accession no.	Amino acid at position												FPV-type
		13	80	87	93	265	267	297	300	305	323	426	555	
HCM-6	AB120720	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HCM-8	AB120721	Ser	Arg	Leu	Asn	Lys	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HCM-18	AB120722	Pro	Arg	Leu	Asn	Thr	Tyr	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HCM-23	AB120723	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HNI-2-13	AB120724	Pro	Arg	Leu	Asn	Thr	Tyr	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HNI-3-4	AB120725	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HNI-3-11	AB120726	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
HNI-4-1	AB120727	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Glu	Val	CPV-2b variant
HNI-1-18	AB120728	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Gly	Tyr	Asn	Asp	Val	CPV-2b
FPV-b CU4[a]	M24004	Pro	Lys	Met	Lys	Thr	Phe	Ser	Ala	Asp	Asp	Asn	Val	FPLV
MEV-d Johnson	M24001	Pro	Lys	Met	Lys	Thr	Phe	Ser	Val	Asp	Asp	Asn	Val	MEV
CPV-b	M38245	Pro	Arg	Met	Asn	Thr	Phe	Ser	Ala	Asp	Asn	Asn	Val	CPV-2
CPV-31	M24000	Pro	Arg	Leu	Asn	Thr	Phe	Ser	Gly	Tyr	Asn	Asn	Ile	CPV-2a
CPV-39	M74849	Pro	Arg	Leu	Asn	Thr	Phe	Ser	Gly	Tyr	Asn	Asp	Val	CPV-2b
V139	AB054222	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Asp	Tyr	Asn	Asn	Val	CPV-2c(a)
V203	AB054224	Pro	Arg	Leu	Asn	Thr	Phe	Ala	Asp	Tyr	Asn	Asp	Val	CPV-2c(b)

[a]FPLV FPV-b CU4, MEV MEV-d Johnson, CPV-2 CPV-b, CPV-2a CPV-31, CPV-2b CPV-39, CPV-2c(a) V139, and CPV-2c(b) V203 were used as reference strains

Table 3. Antigenic characterization of recent parvovirus isolates from Japanese cats and dogs by HI tests

Year of isolation	Isolates				
	FPLV	CPV-2	CPV-2a	CPV-2b	CPV-2b variant
2001	494[a], 495	ND[b]	ND	OM-4, 1074D, 1080D	ND
2002	501, 502, 2045C	ND	2054D	OM-9, OM-10	ND
2003	ND	ND	ND	03-003, MD03-007, MD03-008, MD03-021	ND

[a]Isolates 494, 495, 501, 502, and 2045C were obtained from cats and the other isolates were obtained from dogs

[b]*ND* not detected

typical CPV-2b strains by HI assay. This isolate had no reactivity against MAb B4A2, showing the CPV-2b phenotype (Table 1). However, the reactivity of this isolate against MAb 21C3 was 8 to 32 times lower than that of the other CPV-2b isolates (Table 1). Furthermore, its reactivity against MAb 19D7 [15] was also 8 to 16 times lower than that of the other CPV-2b isolates (Table 1). MAb 19D7 reacts with all antigenic types and recognizes an epitope in the same antigenic site as MAb 21C3 does [15]. Sequence analysis revealed that the residue 426 of the isolate is Glu (Table 2) and suggested that this unique amino acid at the position is responsible for the characteristic antigenicity of the isolate. In addition, this variant is phylogenetically located in the cluster of Vietnamese CPV-2b isolates (Fig. 1). These results indicate that HNI-4-1 is a novel antigenic variant of CPV-2b. Buonavoglia et al. [5] have also reported the Glu-426 variants, strains 56/00 and 136/00, in Italian isolates. They detected these variants by RFLP assay but not by HI assay because CPV-2b-specific MAb (such as MAb 21C3) was not available [5]. Our study first showed that such substitution caused an antigenic difference which could be demonstrated by MAbs.

Glu-426 variants have been overlooked in antigenic analysis using the usual MAbs [5]. Therefore, it is important to re-examine CPV isolates classified into CPV-2b using MAb 21C3. Although we could not detect the Glu-426 variant in recent Japanese CPV-2b isolates (Table 3), a similar evolution may have occurred in CPV-2b. The evidence that Glu-426 variants are independently detected in the distinct areas, Vietnam and Italy, supports this assumption. Further monitoring of field isolates will provide us important information for research on the evolution of CPV.

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Author's address: Dr. Y. Tohya, Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan; e-mail: aytohya@mail.ecc.u-tokyo.ac.jp