

## Persistence of High Virus Neutralizing Antibody Titers in Cats Experimentally Infected with Feline Immunodeficiency Virus

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**ABSTRACT.** The development of virus neutralizing (VN) antibody is one of the most effective host defense mechanisms against virus infection. In the present study, we developed a new VN assay against feline immunodeficiency virus (FIV) using a feline T-lymphoblastoid cell line, MYA-1 cells, based on inhibition of viral reverse transcriptase production. This assay is applicable to strains of FIV which can not infect CRFK cells. By using the assay, we examined long-term responses of VN antibody in cats experimentally infected with FIV. VN antibody titers increased progressively during first 30 weeks post inoculation and remained at high titers thereafter for 7 years of observation periods. — **KEY WORDS:** antibody, FIV, neutralization test.

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The development of virus neutralizing (VN) antibody is one of the most effective host defense mechanisms against virus infection. Thus, it is conceivably important for a better understanding of the pathogenesis of feline immunodeficiency virus (FIV) infection and for future development of vaccine to determine the immune responses in cats against FIV infection.

Several methods for measurement of VN antibody titer against FIV, including inhibition of viral reverse transcriptase (RT) production [2, 6, 13, 21, 27], syncytium formation [2, 4, 13, 23, 24], and FIV p24 or p25 production [2, 3, 28], have been described. In most of these neutralization assays, a feline renal cell line, CRFK cells, was used, and VN antibodies against Petaluma [2–4, 6, 13, 23, 27], Amsterdam 4 and 6 [17, 24], Glasgow 8 [17], Pisa-M2 [2, 13] and Dutch UT113 [3] strains of FIV were measured. However, these assays can be used only for FIV strains which could replicate in CRFK cells. Although Amsterdam 6 and Pisa-M2 strains could not grow initially in CRFK cells, they produced syncytia on infected cells after their adaptation to CRFK cells, and thus the inhibition of syncytium formation was applied for the neutralization assay as a marker [2, 24]. Except two subtype B isolates, KYO-1 and Pisa-M2 [9, 13, 26], all of the strains which have been shown to infect CRFK cells belong to the subtype A based on the sequence analysis [22]. In contrast, most of the subtype B strains including TM1 and TM2 are unable to infect CRFK cells. The neutralization assays using CRFK cells are not applicable to most of the FIV subtype B isolates and probably others. Therefore, it is necessary to use feline T lymphocytes for measurement of VN antibody titer against FIV strains which can not infect CRFK cells.

In this study, we established a new FIV neutralization assay using a FIV-sensitive feline T-lymphoblastoid cell

line, MYA-1 cells, based on the inhibition of viral RT production, and also demonstrated VN antibody responses in cats experimentally infected with FIV.

MYA-1 cells [15] were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 µg/ml of streptomycin, 100 units/ml of penicillin, 50 µM 2-mercaptoethanol, 2 µg/ml of polybrene and 100 units/ml of recombinant human interleukin-2 (rhIL-2) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The rhIL-2 was kindly provided from Dr. M. Hattori (Institute for Immunology, Kyoto University, Kyoto, Japan). CRFK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 100 µg/ml of streptomycin and 100 units/ml of penicillin.

To prepare a stock virus (termed TM219) derived from pTM219, which is a molecular clone of TM2 strain [10], CRFK cells were transfected with 10 µg of pTM219. Two or 3 days after transfection, the culture supernatant from transfected CRFK cells was collected and filtered through 450 nm pore size filter, and then inoculated to MYA-1 cells. Twelve days after inoculation, the culture supernatant from TM219-infected MYA-1 cells was collected and filtered through 450 nm pore size filter, and stored as the stock virus (TM219) at –80°C. TM219 could not infect CRFK cells. Titration of the stock virus was carried out as described previously [8].

Three specific pathogen free (SPF) cats (Cat nos. 19, 24 and 37) were inoculated intraperitoneally with  $3 \times 10^6$  cells of CRFK cells transfected with the pTM219. The inoculum contained approximately  $3 \times 10^4$  of FIV antigen-positive cells when measured by indirect immunofluorescence assay. One SPF cat (Cat no. 15) was inoculated intraperitoneally with  $3 \times 10^6$  cells of uninfected CRFK cells as a negative control. Two SPF cats (Cat nos. 103 and 104) which have been experimentally infected with TM1 and TM2 strains 7 years ago, respectively, were also used. A detailed description on the experimental infection with TM1 and TM2 was previously reported [14].

VN assay was carried out in a 96-well flat bottom

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microplate in quadruplicate. Serum samples were heat-inactivated at 56°C for 30 min prior to VN assay. Fifty  $\mu$ l of serially diluted serum samples (1:8 to 1:4,096) were incubated with 50  $\mu$ l of FIV. Then, 100  $\mu$ l of uninfected MYA-1 cells ( $5 \times 10^5$  cells/ml) were added to this mixture at a total volume of 200  $\mu$ l per well. One day after infection, 100  $\mu$ l of supernatants were removed and 100  $\mu$ l of fresh medium (complete RPMI-1640 as described above) were added. Culture supernatants were harvested at day 12 for RT assay and frozen at  $-80^\circ\text{C}$  until use. VN antibody titer was defined as the reciprocal of the highest serum dilution that gave a 50% or greater reduction of RT activity when compared with virus-infected control cells without serum samples. Virion-associated,  $\text{Mg}^{2+}$ -dependent RT activities in culture supernatant samples were measured as follows; 10  $\mu$ l of culture supernatants were mixed with 50  $\mu$ l of reaction cocktail containing 50 mM Tris-HCl (pH 8.0), 75 mM KCl, 10 mM dithiothreitol, 4.95 mM  $\text{MgCl}_2$ , 0.05% Nonidet P-40, 10  $\mu\text{g}/\text{ml}$  of poly(rA) (Pharmacia Biotech, Uppsala, Sweden) as a template, 5  $\mu\text{g}/\text{ml}$  of oligo(dT)<sub>12-18</sub> (Pharmacia Biotech, Uppsala, Sweden) as a primer and 10  $\mu\text{M}$   $\alpha$ [ $^{32}\text{P}$ ]dTTP. After incubation for 3 hr at 37°C, 10  $\mu$ l of the reaction mixtures were spotted directly onto DEAE ion-exchange paper (DE81, Whatman, Maidstone, U.K.) and washed three times in  $2 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 0.15 M NaCl plus 0.015 M sodium citrate) to remove unincorporated  $\alpha$ [ $^{32}\text{P}$ ]dTTP. For quantitation of RT activity, spots on DE81 paper were counted by a Bio Imaging Analyzer, BAS 2000 (Fuji Photo Film, Tokyo, Japan).

At first, to determine an optimal dose of inoculum for VN assay, three different doses of the virus (10, 100 and 150  $\text{TCID}_{50}/50 \mu\text{l}$ ) were mixed with a serum collected from cat no. 37 at 16 weeks post inoculation (p.i.) and the mixture was incubated for 1 hr. VN antibody titer against 100  $\text{TCID}_{50}/50 \mu\text{l}$  of virus was higher than that against 150  $\text{TCID}_{50}/50 \mu\text{l}$  (data not shown). Due to a low viral dose, 10  $\text{TCID}_{50}$  did not uniformly infect the cells in all wells including control wells without serum samples, and might lead to false positive results. Next, to determine an appropriate incubation time for the mixture of the serum from cat no. 37 and 100  $\text{TCID}_{50}/50 \mu\text{l}$  of virus before addition of MYA-1 cells, 1, 2, 3 and 4 hr of incubation time were examined. VN antibody titers increased with prolongation of the incubation time from 1 to 2 hr. The titers of a 2- or 3-hr incubation were almost the same and higher than that of a 4-hr incubation (data not shown). From

these results, 100  $\text{TCID}_{50}/50 \mu\text{l}$  of virus and a 2-hr incubation time were used for the analysis of VN antibody titer. It has been shown that the sensitivity in VN assays of other lentiviruses, human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), also depends on the virus dose and incubation time [12, 20].

By applying the established conditions of virus dose and incubation time, VN antibody titer in the early stage of FIV infection was measured using sequential serum samples from three SPF cats (Cats nos. 19, 24 and 37) infected with TM219 and one uninfected cat (Cat no. 15). All of the four cats did not have detectable antibody till 8 weeks p.i. Two of the three FIV-infected cats developed VN antibody from 12 weeks p.i. At 16 weeks p.i., VN antibody titers of 1:128 were detected in sera from all of the FIV-infected cats. During observation periods, VN antibody was not detected in sera from the uninfected cat (Table 1). A similar observation was made by Okada *et al.* [16] who detected VN antibody in sera at almost the same time in their superinfection study using FeT cells and Bangston strain of FIV. However, other investigators detected VN antibody activity as early as 4 weeks p.i. [4, 23]. They used CRFK cells and viruses which can infect CRFK cells in their VN assays. Baldinotti *et al.* reported that VN antibody titers against FIV were markedly dependent on passage history of the virus and host cell system [2]. Compared with high-passaged virus in CRFK cells, low-passaged virus in lymphoid cells elicits low VN antibody titers [2]. At the present study, we used low-passaged TM219 prepared from supernatant culturing for only 15 days after transfection. Moreover, the difference in the detectability of VN antibody might be due to several factors, including the origin of FIV and route of FIV infection, amount of virus exposed in cats, assay system, use of  $^{32}\text{P}$  or  $^3\text{H}$  in RT assay, and so on. So, the sensitivity of our assay system was conceivably enough to measure VN antibody titers.

Then, long-term VN antibody responses were also examined using sequential serum samples from cat nos. 103 and 104 infected with TM1 and TM2 strains of FIV,

Table 1. VN antibody titer in the early stage of FIV infection

Cat number	Weeks post inoculation			
	4	8	12	16
15	<8	<8	<8	<8
19	<8	<8	<8	128
24	<8	<8	32	128
37	<8	<8	32	128

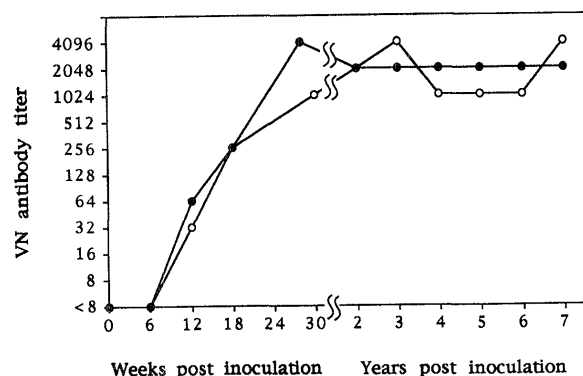


Fig. 1. VN antibody titers of sequential serum samples collected from cats infected with TM1 and TM2 strains of FIV during 7 years of observation periods. Symbols: ○, Cat no. 103 infected with TM1; ●, Cat no. 104 infected with TM2.

respectively (Fig. 1). VN antibody titers could be detected from 12 weeks p.i. and increased progressively. The similar antibody responses in the two cats might be due to the fact that TM1 and TM2 strains are closely related to each other on the basis of genetical and biological properties [11, 14]. High VN antibody titers between 1:1,024 and 1:4,096 were maintained during 7 years p.i. These two cats have exhibited no clinical symptom up to the present, however, viruses were re-isolated from both of the cats at 7 years p.i. (data not shown) and numbers of proviral DNA copy in peripheral blood mononuclear cells (PBMCs) at 6 years p.i. were  $10^{4.0}$  (Cat no. 103) and  $10^{5.7}$  (Cat no. 104) copies per  $10^5$  PBMCs [7]. Recent studies on HIV indicated that high level of virus production and clearance of virus in plasma and infected cells occurred in HIV-infected patients every day [5, 18, 25]. They speculated that the dynamic balance between rapid turnover of viral generation and large amount of viral decay causes the long persistent infection without symptom. Moreover, a correlation between high VN antibody titers and better clinical status was observed in HIV and SIV studies [1, 12, 19]. Our findings suggest that the persistence of high level of VN antibody might be induced by continuous production of virus and inhibit the disease progression. Recently, Matteucci *et al.* reported that even in the absence of detectable VN antibody in vaccinated cats, the protection against challenge was observed [13]. Further investigations will be required to clarify the role of VN antibody in FIV-infected cats. This is the first report on the long-term VN antibody response in cats infected with FIV subtype B, and the present VN assay system may be useful for studies of immune responses in cats against FIV infection, cross neutralization among FIV subtypes, and vaccine development.

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