Evidence for CD8⁺ Antiviral Activity in Cats Infected with Feline Immunodeficiency Virus

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Human immunodeficiency virus (HIV) causes a long, asymptomatic infection characterized by normal to elevated numbers of circulating CD8⁺ cells and a progressive decline in CD4⁺ cells. It has been speculated that HIV-specific antiviral activity driven by CD8⁺ T cells may control viral replication during this period and maintain the clinically asymptomatic stage of disease. The disease induced in cats by feline immunodeficiency virus (FIV) is similar to HIV in that it is characterized by a long asymptomatic stage with a progressive decline in CD4⁺ cells, culminating in AIDS. In the present study, we demonstrate that FIV is more readily isolated from CD8⁺ T-cell-depleted peripheral blood mononuclear cells (PBMC) of FIV-infected cats than from unfractionated PBMC cultures. In addition, CD8⁺ T cells isolated from FIV-positive cats demonstrating anti-FIV activity in PBMC cultures inhibit FIV infection of FCD4E cells in vitro. Anti-FIV activity is not found in FIV-negative cats and is not characteristic of cats acutely infected with FIV but is present in the majority of chronically infected, clinically asymptomatic and symptomatic cats. Decreases in plasma and cell-associated viremia during the acute-stage FIV infection appears to precede the appearance of CD8⁺ anti-FIV cells in the circulation. In summary, this study demonstrates a population(s) of CD8⁺ T cells in chronically FIV-infected cats capable of suppressing FIV replication in cultured PBMC. The significance of anti-FIV CD8⁺ cells in the immunopathogenesis of the infection and disease progression has yet to be determined.

Human immunodeficiency virus (HIV), a member of the lentivirus subfamily of retroviruses, is thought to be the etiologic agent of human AIDS. The pathogenesis of HIV-induced AIDS has been attributed to virus-induced reduction in T helper (CD4⁺) cell numbers and function, resulting in gradual deterioration of immune competence and development of severe secondary infections (7, 8).

During the early acute stage of HIV infection, there are a burst of virus replication and high levels of plasma viremia (8). The acute-stage infection is followed by a period of clinical latency, during which the numbers of infected cells in the blood are low, and it is difficult to detect virus in the plasma. Although recent studies have indicated that replication of HIV in vivo is continuous and productive, even during the clinically latent period (11, 30), other studies have supported the hypothesis that a subset of circulating CD8⁺ cells is capable of inhibiting HIV replication in CD4⁺ cells and reducing plasma viremia during the asymptomatic stage of disease. Walker et al. (27, 29) found that virus expression by infected CD4⁺ cells in peripheral blood mononuclear cells (PBMC) from asymptomatic individuals was downregulated by CD8⁺ cells. Removal of these CD8⁺ cells led to increased virus replication. Similar simian immunodeficiency virus-specific antiviral cells in the circulating PBMC of monkeys have been described (12, 21). In support of these studies, Kannagi et al. (13) demonstrated that peripheral blood lymphocytes from asymptomatic HIV type 1 (HIV-1) carriers do not support replication of exogenously added HIV-1. This interference was dependent on the presence of CD8⁺ lymphocytes in the culture. Such resistance is

not seen in patients with advanced disease, suggesting that a deterioration in CD8⁺ antiviral activity may contribute to development of clinical disease. Thus, one of the major unanswered questions regarding the immunopathogenesis of HIV-1 infection is what maintains the long asymptomatic stage of disease despite active virus replication and a rapid turnover of infected CD4⁺ lymphocytes.

Feline immunodeficiency virus (FIV), a lentivirus of cats, is associated with feline AIDS (20). What is known to date of the pathogenesis of FIV infection suggests that it is a valuable animal model for HIV-induced AIDS (9). HIV and FIV have similar morphologies, protein compositions, and Mg²⁺ dependencies of their reverse transcriptases (RT) (18). Both viruses preferentially infect CD4⁺ T lymphocytes, leading to an inversion of the CD4⁺/CD8⁺ lymphocyte ratio (1, 6, 24, 26).

The clinical disease associated with FIV also parallels that seen in HIV infection. Four to 6 weeks following experimental infection with FIV, cats develop an acute infection syndrome manifested in fever and lymphadenopathy, similar to that seen with HIV-1. This is followed by a long asymptomatic period in which the CD4⁺/CD8⁺ ratio progressively declines and immune function deteriorates (6, 31). During this time, it is difficult to detect infectious virus in the plasma despite the relatively high levels of FIV mRNA (4). English et al. (6) recently reported that cats experimentally infected with FIV developed clinical signs of AIDS approximately 18 to 36 months postinfection (p.i.) and that disease development correlated with a decrease in the CD8⁺ cell numbers. In this study, we explored the possibility that CD8⁺ cells that downregulate FIV expression may exist in FIV-infected cats and play a role in maintaining the long asymptomatic stage of disease.

MATERIALS AND METHODS

Experimental animals. Six naturally and 35 experimentally FIV-infected cats were used in this study. The experimentally infected cats consisted of 5 random-source (RS) (local licensed dealer) and 30 specific-pathogen-free (SPF) (Liberty

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FIG. 1. Mg^{2+} -dependent RT activity in PBMC from naturally infected cats. PBMC from six naturally FIV-infected cats, BIG (▲), CIN (◆), THO (+), TOM (●), ROM (▼), and JOS (■), were cocultured with indicator FCD4E cells as described in Materials and Methods. Culture supernatant was collected for RT activity at intervals postculturing. Data are the means $\pm$ standard errors (SE) of triplicates; the RT activity of culture supernatant from FCD4E cells alone was 191 ± 20 cpm. Some error bars are smaller than the markers and do not appear in the figure.

Labs, Liberty, N.J.) cats. Prior to infection, all animals were negative for feline leukemia virus antigen by enzyme-linked immunosorbent assay (ELISA) (TechAmerica, Omaha, Nebr.) and negative for antibody to FIV as determined by Western blotting (immunoblotting). The six naturally infected cats were negative for feline leukemia virus antigen and positive for FIV antibody by ELISA (IDEXX, Portland, Maine). All the cats were housed in the North Carolina State University College of Veterinary Medicine Laboratory Animal Care Facility during the experimental period. The clinical states of these cats, CD4⁺/CD8⁺ ratios, and virus expression have been described previously (6, 24).

Experimental FIV infection of cats. The NCSU₁ isolate of FIV (FIV-NCSU₁) was originally isolated from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described previously (6). Experimentally infected cats were inoculated with either 2×10^6 PBMC from the FIV-NCSU₁ donor cats (6) or 6×10^5 50% tissue culture infective doses (TCID₅₀) of cell-free virus (3). FIV infection was confirmed by the presence of serum antibody to FIV by ELISA (IDEXX) and Western blotting (19), the presence of a high level of Mg^{2+} -dependent RT activity in the PBMC (6), and positive PCR for proviral DNA in PBMC (5).

PCR. FIV provirus was detected by PCR and Southern blot analysis of *gag* sequences as described by English et al. (5).

Lymphocyte subset analysis. Blood was collected for a complete blood count and flow cytometric analysis. Cells were purified and prepared for flow cytometric analysis as previously described (19, 23), and lymphocyte subsets were identified by using monoclonal antibodies 1.572 (Pan T), 3.357 (CD8⁺), and CAT30A (CD4⁺) (23). A fluorescein isothiocyanate-conjugated F(ab')₂ fragment of a goat anti-cat immunoglobulin G heavy- and light-chain-specific antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used to identify B cells. The percent positively stained lymphocytes was determined by flow cytometric analysis using a Becton Dickinson FACScan. Complete blood counts were performed on a Coulter Counter (Coulter, Hialeah, Fla.), and absolute lymphocyte numbers were determined from a differential cell count.

Separation of CD8⁺ T cells from whole blood. Blood was collected in EDTA and PBMC purified over a Percoll gradient as previously described (25). CD8⁺ T cells were enriched from PBMC by using monoclonal antibody 3.357 (anti-cat CD8⁺) and M450 magnetic beads (Dynal, Great Neck, N.Y.) as described by English et al. (5). PBMC (10^7) were washed twice with 2% fetal bovine serum-phosphate-buffered saline, incubated in 1 ml of anti-CD8 monoclonal antibody for 30 min at 4°C, washed, and then incubated with 1 ml of M450 beads that had been coated with goat anti-mouse immunoglobulin G. The ratio of cell to beads was 1:3. After the bead-bound cells (CD8⁺ T cells) were magnetically removed, the remaining cells (CD8⁻ PBMC) were resuspended in medium, gently agitated to release any trapped nonbound cells, and then magnetically reisolated. After double magnetic sorting, the bead-free cells were <2% CD8⁺ as determined by flow cytometric analysis. The CD8⁺ population released its magnetic beads after 48 to 72 h in culture, with complete reexpression of the CD8 molecule by 5 days. These cells were >98% positive for CD8 after 6 days in culture.

In vitro coculture for FIV expression. Regulation of FIV expression by CD8⁺ T cells was studied by coculture of PBMC and CD8⁺-depleted PBMC with the highly FIV-susceptible, CD4⁺ feline lymphocyte culture FCD4E (5). PBMC were incubated in 48-well culture plates for 3 h with 5 μ g of concanavalin A (ConA) per ml, washed, and then incubated for 24 h in the presence of 100 U of recombinant human interleukin 2 (rHuIL-2) per ml. This incubation period was

chosen because Rottman et al. (22) demonstrated peak cytokine levels 6 to 12 h after stimulation of feline PBMC with ConA, and we suspected that the antiviral activity was mediated by cytokines. Following stimulation, CD8⁺ T cells were cocultured with FCD4E cells which had been infected 16 h earlier with a multiplicity of infection of 0.01 TCID₅₀ of FIV-NCSU₁ in ConA-free medium containing 100 U of rHuIL-2 per ml at a CD8⁺ T-cell-to-FCD4E cell ratio of 4:1. In all cultures, the final cell concentration was 2×10^6 /ml. During the culture, half of the medium was removed every 3 or 4 days and stored at -79°C for subsequent RT assay. An equal amount of fresh medium was added to the cultures at this time.

In vitro infection of FCD4E cells with FIV. Cells were infected in vitro with cell-free virus from FCD4E cells prepared as described by English et al. (5). Briefly, 10^7 FCD4E cells, which were pretreated with 25 μ g of DEAE-dextran per ml for 30 min at room temperature, were incubated with 10^5 TCID₅₀ of FIV-NCSU₁ for 2 h at 37°C. The cells were then washed twice and resuspended in fresh culture medium for CD8⁺ inhibition studies.

RT assay. An assay for Mg^{2+} -dependent RT activity was performed as described previously (19) and is a modification of the procedure described by Goff et al. (10). Triplicate measurements were made for each supernatant sample.

Cell-associated viremia. At various times p.i., PBMC from eight SPF cats infected at 12 weeks of age with 6×10^5 TCID₅₀ of cell-free virus were cocultured with FCD4E cells. In one well of a 24-well plate, 5×10^5 PBMC from an infected cat and 5×10^5 FCD4E cells were cultured in 1 ml of RPMI medium containing 10% fetal bovine serum, 100 U of rHuIL-2 per ml, and 5 μ g of ConA per ml. On culture days 7, 14, and 21, half of the coculture supernatant was removed and frozen at -135°C for determination of RT activity. The cultures were replenished with fresh medium without ConA. For each group of cocultures, FCD4E cells inoculated with 10, 1, 0.1, and 0.01 TCID₅₀ of virus stock served as positive controls, and uninfected FCD4E cells and FCD4E cells cocul-

FIG. 2. RT activity in CD8⁺ cell-depleted PBMC from naturally infected cats. (A) TOM; (B) ROM. PBMC (▲) or CD8⁺ T-cell-depleted PBMC (●) were cocultured with FCD4E cells (indicator cells) in a 1:1 ratio (total, 2×10^6 cells per ml) in the culture. CD8⁺ T-cell-depleted PBMC were obtained as described in Materials and Methods. The RT level of the negative control was 99 ± 9 cpm. Data are means $\pm$ SE of triplicates. Some error bars are smaller than the markers and do not appear in the figure.

TABLE 1. FIV replication in unfractionated and CD8⁺ cell-depleted PBMC from cats infected with FIV

Cat ^a	Peak RT activity in culture supernatant (10 ² cpm/μl) ^b		% Suppression ^c
	Unfractionated PBMC	CD8 ⁺ cell-depleted PBMC	
BIG	65.5 ± 1.5	59.2 ± 1.5	
CIN	90.0 ± 2.8	57.3 ± 4.6	
JOS	1.1 ± 2.8	0.5 ± 0.01	
ROM	0.6 ± 0.1	26.1 ± 1.5	92.8
THO	31.0 ± 3.2	63.8 ± 2.0	67.1
TOM	0.7 ± 0.04	76.0 ± 2.6	99.7

^a The cats were naturally infected with FIV as determined by Western blotting for anti-FIV antibody and PCR for FIV provirus in PBMC.

^b Data are peak RT activities for each culture (means ± SE from triplicate cultures) of PBMC or CD8⁺ T-cell-depleted PBMC cultured with FCD4E indicator cells. RT assays were run in triplicate for each supernatant sample.

^c Expressed as: 100 – (RT activity of unfractionated PBMC culture/RT activity of CD8⁺ T-cell-depleted culture). Cats with ≥66% suppression were arbitrarily defined as having CD8⁺ anti-FIV activity. Coculture methods and depletion of CD8⁺ T cells from PBMC and FCD4E cells are described in Materials and Methods.

tured with PBMC from an uninfected SPF cat served as negative controls. All cultures were performed in duplicate.

To decrease overall assay variation, 7-, 14-, and 21-day culture supernatants from a single p.i. time point were assayed together. Magnesium-dependent RT activity was always greatest in the 21-day coculture supernatants. Therefore, the RT activity of each coculture at 21 days was used as a relative measure of viral expression.

Plasma viremia. The expression of virus by PBMC in culture may not reflect the level of viral replication *in vivo*. Therefore, at various times p.i., the level of infectious virus in the plasma of FIV-infected cats was also measured. At each time p.i. that cell cocultures were performed, EDTA-treated plasma from the eight cats was also collected and used to inoculate FCD4E cells. Previous experiments demonstrated that EDTA-treated plasma was highly toxic to FCD4E cells. However, this toxicity was not observed if calcium was added to the plasma to a level that resulted in clot formation. Plasma was frozen at –135°C until assayed, allowing for samples from all cats at each time postinoculation to be cultured simultaneously to eliminate interassay variation. For each sample, 20 μl of 0.1 M CaCl₂ was added to 125 μl of EDTA-treated plasma and the mixture was allowed to clot for 40 min at 4°C. The clotted plasma samples were then centrifuged, and 50 μl of the resultant serum was added to 2 × 10⁵ FCD4E cells in 100 μl of medium in a 96-well round-bottom plate. At various times postculture, supernatant samples were removed and frozen for determination of FIV p24 antigen levels, and the cultures were replenished with fresh medium. All cultures were performed in duplicate. FCD4E cells inoculated with 10, 1, 0.1, and 0.01 TCID₅₀ of virus stock served as positive controls, and uninfected FCD4E cells and FCD4E cells inoculated with plasma from an SPF control cat served as negative controls.

A commercially available p24 FIV antigen detection ELISA kit (IDEXX) became available as these experiments were being performed. Preliminary experiments in our laboratory indicated that the antigen detection kit was more sensitive than the RT assay in detecting viral replication. Therefore, ELISA antigen detection was used as a measure of viral replication instead of RT activity for the plasma cocultures.

RESULTS

Effect of CD8⁺ depletion on FIV RT expression in cultured PBMC. Walker et al. (29) reported that depletion of CD8⁺ cells from cultured PBMC of asymptomatic HIV-infected individuals resulted in enhanced expression of HIV RT activity in coculture. Diehl et al. (4) reported that after an acute-stage infection characterized by high levels of FIV mRNA in the plasma, levels of FIV mRNA decrease by 2 to 3 log units during the asymptomatic stage of infection. In addition to other potential mechanisms, such as neutralizing antibody, it is possible that expression of FIV in feline PBMC during the asymptomatic infection is downregulated by inhibitory CD8⁺ cells.

To address this question, we studied a group of cats naturally

infected with FIV. These cats had been infected for at least 2 years, and all were asymptomatic at the time of the study. All cats were shown to be infected with FIV by Western blotting for anti-FIV antibody and by PCR for FIV provirus in PBMC (data not shown). To establish baseline RT activity in these cats, 10⁶ PBMC were cocultured with FCD4E indicator cells, and Mg²⁺-dependent RT activity was measured at intervals for 16 days. The results in Fig. 1 show that PBMC from three cats (BIG, CIN, and THO) produced significant amounts of RT activity in coculture. Three cats (TOM, ROM, and JOS) failed to produce significant levels of RT even after 16 days (Fig. 1).

To examine the influence of CD8⁺ cells on FIV expression by PBMC from these cats, CD8⁺ lymphocytes were removed with anti-CD8⁺ antibody-coated magnetic beads. The CD8⁺ cell-depleted population, containing <2% CD8⁺ cells as determined by flow cytometry analysis (data not shown), and unfractionated PBMC were cocultured with FCD4E cells. Because CD8⁺ cells were not immediately released from the magnetic beads, it was not possible to do CD8⁺ reconstitution experiments. The reproducibility of virus expression by PBMC from each cat is evidenced from the observation that the patterns of FIV RT expression by unfractionated PBMC from the different cats were the same in experiments 1 and 2 (see Table 2) (Fig. 2). Furthermore, the two cats (ROM and TOM) that revealed the lowest RT activity in unfractionated PBMC (Fig. 1) also provided the strongest evidence of CD8⁺ suppressor activity (Fig. 2). Removal of CD8⁺ cells from the PBMC resulted in a significant enhancement of RT activity in coculture with regard to both time in culture and magnitude of the signal (Fig. 2). PBMC from the two cats that expressed the greatest RT activity in unfractionated PBMC (CIN and BIG) showed no increase in RT levels following CD8⁺ depletion (Table 1).

TABLE 2. In vitro FIV expression by PBMC and CD8⁺ cell-depleted PBMC from experimentally FIV-infected cats

Cat ^a	Peak RT activity in culture supernatant (10 ² cpm/μl) ^b		% Suppression ^c
	Unfractionated PBMC	CD8 ⁺ cell-depleted PBMC	
AL1	10.7 ± 3.6	109.9 ± 0.1	90.3
AL2	47.2 ± 1.7	102.6 ± 3.8	53.9
AL3	1.4 ± 0.1	1.3 ± 0.2	
AL4	1.1 ± 0.1	71.8 ± 3.7	98.6
AL5	12.7 ± 1.9	58.6 ± 2.7	78.3
AL6	64.4 ± 5.2	29.3 ± 2.8	
AN3	6.2 ± 1.4	10.9 ± 3.6	43.1
AN4	3.7 ± 0.3	41.5 ± 3.4	91.1
AN5	6.2 ± 0.4	109.3 ± 2.2	94.3
AN6	2.7 ± 0.8	7.4 ± 2.1	63.5
AK1	5.5 ± 0.5	37.1 ± 1.8	85.2
AK2	33.3 ± 4.0	74.0 ± 3.6	55.0
AK3	1.0 ± 0.3	0.9 ± 0.1	
AK4	0.5 ± 0.1	28.4 ± 2.0	98.2
AK5	17.5 ± 0.3	56.9 ± 2.4	69.2

^a AL and AN SPF cats were tested 44 to 60 weeks p.i. with cell-free FIV and were clinically asymptomatic at the time of testing. AK RS cats were tested 95 weeks p.i. with cell-associated virus. Some of the AK cats had clinical signs of early ARC disease (chronic oral and recurrent respiratory infections) around the time of testing.

^b Data are mean peak RT activities (± SE) of triplicate samples (16 days postcoculture) from cultures of PBMC or CD8⁺ cell-depleted PBMC with indicator FCD4E cells.

^c Expressed as: 100 – (RT activity of unfractionated PBMC culture/RT activity of CD8⁺ T-cell-depleted culture). Coculture methods and depletion of CD8⁺ T cells from PBMC and FCD4E cells are described in Materials and Methods.

TABLE 3. Inhibitory effect of CD8⁺ cells from FIV-infected cats on in vitro infection of FCD4E cells

Cat/FIV status	% Suppression of FIV replication in FCD4E cells ^a	Previous antiviral activity ^b
AL4/positive	90.2	98.6
AL5/positive	92.3	78.3
AL6/positive	<10	0
AN6/positive	87.0	63.5
AN3/positive	39.7	43.1
AN4/positive	38.3	91.1
AN5/positive	38.3	94.3
TK2/negative	<10	NT ^c
TJ2/negative	<10	NT

^a CD8⁺ cells were removed with anti-CD8 antibody-coated magnetic beads from PBMC of seven FIV-infected cats shown previously to have various degrees of antiviral activity and from PBMC of two FIV-negative cats. After stimulation with ConA and rHuIL-2 as described in the text, the CD8⁺ cells were added to FCD4E cultures which had been infected 16 h previously with FIV as described in Methods and Materials. RT activity was measured after 9 days in culture.

^b Data are previous antiviral activities determined by CD8⁺ cell-depleted PBMC cocultures and are from Table 2 (expressed as percent suppression).

^c NT, not tested.

One cat (JOS) showed no evidence of FIV replication in the presence or absence of CD8⁺ lymphocytes (Table 1). As we were unable to obtain high enough RT levels from CD8⁺ cell-depleted PBMC or unfractionated PBMC without coculturing with FCD4E cells, we were not able to determine whether the increased RT in CD8⁺ cell-depleted cultures arises from increased expression in PBMC or a spread to FCD4E cells. We are currently addressing these possibilities by using p24 as a measure of virus expression, which we believe will be more sensitive than the RT assay and may allow measurements of virus in PBMC without coculturing.

CD8⁺ cell-mediated anti-FIV activity in cats experimentally infected with FIV. To further investigate CD8⁺ antiviral activity in a more controlled setting, the effect of CD8⁺ depletion in PBMC from cats experimentally infected with FIV-NCSU₁ was measured. Ten SPF cats had been infected for approximately 44 to 60 weeks (AL and AN cats; Table 2), and five RS cats had been infected for 95 weeks (AK cats; Table 2). While the SPF cats were clinically asymptomatic at this time, despite having inverted CD4⁺/CD8⁺ ratios (data not shown), the RS cats were beginning to show evidence of early-stage AIDS-related complex (ARC) disease, including recurrent oral and respiratory infections. Depletion of CD8⁺ cells from PBMC resulted in enhanced FIV expression in 5 of the 10 SPF cats infected with FIV for 44 to 60 weeks (Table 2). Three of the five RS cats infected for 95 weeks showed evidence of CD8⁺ cell-mediated anti-FIV activity (Table 2). Thus, anti-FIV CD8⁺ cell activity appears to be a characteristic of the PBMC of approximately 50% of cats with experimental FIV infection. This is similar to the frequency of CD8⁺ antiviral activity in cats naturally infected with FIV and with clinically latent disease (Table 1). Interestingly, the first group of cats (AL and AN) in this study was infected with cell-free virus, whereas the second group (AK) was infected with cell-associated virus, indicating that the source of virus and the source of cat (random source or SPF) were not factors in development of CD8⁺ antiviral activity.

Inhibitory effect of CD8⁺ cells on FIV replication in cultured FCD4E cells. One interpretation of the data in Fig. 2 is that CD8⁺ cells may downregulate FIV expression in PBMC of cats with asymptomatic infections. Kannagi et al. (13) reported that CD8⁺ cells from HIV-infected individuals inhibited in vitro infection of CD4⁺ cells by exogenous HIV-1. In this experiment, we evaluated FIV-infected cats that had been

shown in previous experiments to have various levels of antiviral activity (Table 2) and two control cats for evidence of CD8⁺ inhibition of FIV replication in cultured FCD4E cells. CD8⁺ cells were separated from PBMC on antibody-coated magnetic beads as described above. The CD8⁺ cells eluted from the beads were incubated with 5 µg of ConA per ml for 3 h, washed, and incubated with 100 U of rHuIL-2 per ml. Twenty-four hours later, the cells were washed and added to FCD4E cells that had been infected with FIV 16 h earlier. The results in Table 3 demonstrate that purified CD8⁺ cells from six of seven FIV-infected cats had some ability, and those from three cats had significant (≥66%) ability, to suppress FIV replication in FCD4E cells. The one cat (AL6) that showed no evidence of CD8⁺ anti-FIV activity in PBMC (Table 2), as well as CD8⁺ cells from normal cats, failed to show evidence of CD8⁺ inhibition of FIV replication in FCD4E cells (Table 3).

CD8⁺ antiviral activity in cats with different stages of FIV infection. CD8⁺ antiviral activity in HIV-infected individuals appears during the acute-stage infection, persists during the asymptomatic stage of disease, and deteriorates in individuals

TABLE 4. CD8⁺ T-cell antiviral activity in PBMC from cats with different stages of FIV infection

Stage of infection and cat	Peak RT activity in culture supernatant (10 ² cpm/10 µl)		Wk p.i.	% Suppression ^a
	Unseparated PBMC	CD8 ⁺ cell- depleted PBMC		
Acute ^b				
AL1 ^c	8.2	7.6	7	0
AL2 ^c	9.4	17.2	7	45.3
AL3 ^c	10.7	13.8	7	22.5
AM1	105.8	77.3	13	0
AM2	29.9	33.3	13	10.2
AM3	33.5	23.0	13	0
AM4	58.7	31.5	13	0
AM5	54.7	26.4	13	0
Asymptomatic ^b				
AP2	169.1	225.2	22	24.9
AP3	20.7	32.1	22	35.5
AP4	9.6	148.9	22	93.6
AP5	16.3	66.1	22	75.3
AL1 ^c	10.7	109.9	44	90.3
AL2 ^c	47.2	102.6	44	54.0
AL3 ^c	1.03	22.1	44	95.5
AL4	1.1	71.8	44	98.5
AL5	12.7	58.6	44	78.3
AL6	64.4	29.3	44	0
Asymptomatic-ARC ^d				
AK1 (ARC)	5.5	37.1	95	85.2
AK2 (ARC)	33.3	74.0	95	55.0
AK3 (ARC)	1.0	0.9	95	0
AK4	0.5	28.4	95	98.2
AK5 (ARC)	17.5	56.9	95	69.2
AN3	6.2	109.3	105	94.3
AN4	3.7	41.5	105	91.1
AN5	58.0	40.0	105	0
AN6	2.7	7.4	105	63.5

^a Expressed as: 100 - (RT activity of unfractionated PBMC culture/RT activity of CD8⁺ T-cell-depleted culture).

^b SPF cats were infected with cell-free FIV as described in Materials and Methods.

^c Cats AL1, AL2, and AL3 were tested at both the acute and the asymptomatic stages of infection.

^d Asymptomatic-ARC cats consisted of RS cats (AK) infected with cell-associated virus and SPF cats (AN) infected with cell-free virus. Four of the AK cats (ARC) had chronic oral and recurrent respiratory infections around the time of testing.

FIG. 3. Cell- and plasma-associated viremia in SPF cats inoculated with 5×10^6 TCID₅₀ of FIV-NCSU₁. (A) Cell-associated virus in PBMC during the acute phase of FIV infection. Data are the mean 21-day RT activities (triplicate assays) in PBMC cocultures of samples collected at various times p.i. from eight FIV-infected SPF cats. Data for individual cats (solid lines) and the mean 21-day RT activity in a PBMC coculture of a sample from an FIV-negative cat (dashed line) are shown. (B) Plasma-associated viremia. Fifty microliters of plasma collected at various times p.i. were used to inoculate FCD4E cells, and culture supernatants were assayed by ELISA for p24 levels 21 days later. Data for individual FIV-infected cats (solid lines) and optical density (O.D.) readings for culture supernatants of plasma from an FIV-negative cat (dashed line) are shown.

with AIDS (7, 29). To address the questions of time of induction and duration of this activity with progression of FIV infection, we examined CD8⁺ cell-mediated antiviral activity in three groups of cats with different stages of FIV infection: acute stage (7 to 13 weeks p.i.), early asymptomatic stage (22 to 44 weeks p.i.), and late asymptomatic stage (95 to 105 weeks p.i.), at which time some cats had ARC. The 10 asymptomatic FIV-infected cats (22- to 44-week-p.i. group) are represented by 4 cats (AP group) infected for 22 weeks plus the AL group described in Table 2 and infected for 44 weeks. The asymptomatic-ARC group (95 to 105 weeks p.i.) consists of the five FIV-infected RS cats described in Table 2 (AK group) and four FIV-infected SPF cats (AN group) that had been infected for approximately 105 weeks. Four of the five FIV-positive RS cats showed evidence of ARC, as manifested in chronic oral and recurrent respiratory infections.

In total, 11 of 27 cats showed evidence of CD8⁺ anti-FIV activity ($\geq 66\%$ suppression), as measured by increased RT activity in CD8⁺-depleted PBMC compared with that in un-

fractionated PBMC (Table 4). None of eight cats tested during the acute stage of infection (7 to 13 weeks p.i.) showed evidence of CD8⁺ antiviral activity (Table 4). In contrast, 6 of 10 cats tested at 22 to 44 weeks p.i. (asymptomatic stage) showed significant CD8⁺ anti-FIV activity ($\geq 66\%$ suppression) (Table 4). Five of nine cats with long-term infection (95 to 105 weeks p.i.) demonstrated significant CD8⁺ anti-FIV activity. There appeared to be no relationship between CD8⁺ antiviral activity and clinical condition, as four of nine cats in this group, including two with antiviral activity (AK1 and AK5), had evidence of clinical disease (chronic oral and respiratory infections), whereas none of the cats with the early-stage asymptomatic infection (22 to 44 weeks p.i.) had clinical evidence of FIV-associated disease. The clinical disease in these cats has recently been described (6). In addition, CD8⁺ antiviral activity in the asymptomatic-ARC group of cats was equally distributed between SPF (AN3 and AN4) and RS (AK1, AK4, and AK5) cats. Also of interest, cats AL1 and AL3, which showed no evidence of antiviral activity when tested at 7 weeks p.i., showed strong CD8⁺ cell-mediated anti-FIV activity when tested at 44 weeks p.i.

Kinetics of virus expression during acute FIV infection. If CD8⁺ cell-mediated antiviral activity plays a role in downregulating virus expression, one would predict that the appearance of CD8⁺ anti-FIV activity during the acute-asymptomatic transition stage of disease (Table 4) would correlate with a decrease in cell-associated and/or plasma viremia. To determine if this was the case, cats with acute-stage infection were bled at intervals for cell-associated and plasma virus assays as described in Materials and Methods. These cats were infected with the same dose of cell-free FIV as those with acute-stage and asymptomatic-stage infections listed in Table 4.

As shown in Fig. 3A, cell-associated virus, as measured by RT activity, peaked in cocultures of PBMC taken at 2 and 4 weeks p.i. By 6 weeks p.i., RT activity in PBMC cocultures had markedly decreased in seven of the cats. High-level RT activity was detected in cocultures of PBMC from the remaining cat up to 11 weeks p.i. These findings demonstrate that a high level of in vitro virus expression by PBMC collected 2 to 4 weeks after infection was followed by a rapid decrease in viral recovery from PBMC by 6 weeks p.i. Virus levels remained low when tested at 11 and 15 weeks p.i.

All eight cats had significant plasma viremia at 2 weeks p.i. (Fig. 3B). Supernatant antigen levels peaked after 18 to 21 days in culture and correlated with syncytium formation and cell lysis. A low level of viremia was also present at 4 weeks p.i. in plasma samples from several cats. However, infectious virus was not recovered from plasma samples taken preinfection or after 4 weeks p.i. These findings paralleled the cell-associated viremia results and indicate that after an initial burst of rapid replication and release of infectious virus, the level of circulating infectious virus in FIV-infected cats dramatically decreases. At no time p.i. could antigen be directly detected in plasma with the ELISA (data not shown). Thus, cell-associated and plasma virus titers appeared to decline prior to the appearance of CD8⁺ antiviral activity (Table 4).

DISCUSSION

The acute stage of HIV-1 infection in humans, characterized by a high level of plasma viremia, is followed by a long asymptomatic stage manifested by relatively stable numbers of CD4⁺ and CD8⁺ cells and low levels of detectable virus. It has been speculated that low-level virus expression during the clinically asymptomatic stage of disease is due to an active inhibition of HIV replication by a population of CD8⁺ cells capable of

suppressing HIV replication (13, 29). This inhibition of virus production has been detected by depletion of CD8⁺ cells from PBMC from HIV-positive patients and by inhibition of in vitro HIV infection of CD4⁺ cells by CD8⁺ cells from HIV-positive patients (27).

Natural as well as experimental FIV infections of cats also result in a long asymptomatic stage characterized by an inverted CD4⁺/CD8⁺ ratio and a low virus burden in PBMC (6, 31). In this study, we demonstrate that cultures of CD8⁺ cell-depleted PBMC from cats with either natural or experimental FIV infections express markedly more FIV RT activity than unfractionated PBMC cultures (Tables 1, 2, and 4). Our results show that, in addition to suppressing FIV expression by in vivo-infected PBMC, CD8⁺ cells from FIV-infected cats, but not those from noninfected cats, were capable of inhibiting FIV infection of susceptible FCD4E lymphocyte cultures in vitro (Table 3). In a limited number of cats, there was a correlation between CD8⁺ inhibition of FIV expression by in vivo-infected PBMC and in vitro-infected FCD4E cells. These observations are consistent with reports that CD8⁺ cells are able to suppress HIV replication by PBMC from HIV-infected patients as well as inhibit in vitro FIV infection of CD4⁺ cell lines (13, 27).

For HIV-infected individuals, CD8⁺ antiviral activity has been consistently demonstrated for patients with asymptomatic infections but not ARC or AIDS patients (13, 29). The data reported here indicate that some, but not all, cats with asymptomatic FIV infection, as well as those with clinical evidence of ARC, possess CD8⁺ anti-FIV activity. For example, two of four cats with FIV-associated clinical diseases, including chronic gingivitis, weight loss, upper respiratory disease, and ocular lesions, demonstrated effective in vitro CD8⁺ anti-FIV activity, whereas in two separate experiments, 9 of 15 cats (Table 4) with asymptomatic FIV infection showed evidence of CD8⁺ anti-FIV activity. It is not possible to draw a correlation between expression of CD8⁺ virus-inhibitory activity and the disease status of the FIV-infected cats until larger numbers of FIV-positive cats with clinical disease are available for study. Also, it should be stressed that these cats appeared to be experiencing early-stage ARC rather than the clinically more serious AIDS diseases characteristic of later-stage infection.

Mackewicz et al. (16) studied a group of HIV-positive patients through the acute and asymptomatic infection stages and reported that CD8⁺ anti-HIV activity developed relatively early in the acute-asymptomatic transition stage of disease. The temporal relationship between the appearance of CD8⁺ anti-FIV activity and disease progression was also investigated in this study. None of the 8 cats studied during the acute stage of infection (Table 4) demonstrated CD8⁺ anti-FIV activity, whereas a large fraction (6 of 10 cats) at 22 to 44 weeks p.i. (early asymptomatic stage) demonstrated CD8⁺ antiviral activity (Table 4). Of interest in this study was the observation that two cats that showed no evidence of CD8⁺ cell-mediated antiviral activity when tested during the acute stage of infection (Table 4) possessed strong antiviral activity when retested at the early asymptomatic stage of infection (Table 4). Thus, similar to the case for HIV patients, anti-FIV activity probably develops during the acute-to-asymptomatic transition stage of the disease.

Diehl et al. (4) measured high levels of virus mRNA expression in the circulation of experimentally infected cats during the acute phase of infection, but virus levels declined during the asymptomatic phase. Although it is possible that induction of CD8⁺ anti-FIV cells early after infection does play a role in downregulating FIV expression, our data (Table 4) do not demonstrate a high frequency of CD8⁺ antiviral activity until

the asymptomatic stage of infection, well after cell-associated and plasma viremia have declined significantly (Fig. 3). Similarly, Mackewicz et al. (17) detected CD8⁺ T-cell noncytolytic anti-HIV activity in the PBMC of seven acute-stage HIV-positive patients, but only two patients showed a corresponding decrease in plasma virus titers. Additional studies will be necessary to establish a relationship between CD8⁺ virus suppressor cells and control of virus expression in vivo. Thus, it will be important to explore other potential mechanisms of regulating FIV and HIV expression, including neutralizing antibody, cytotoxic CD8⁺ or NK cells, and nonimmunological mechanisms.

How CD8⁺ cells suppress FIV expression in PBMC or cultured FCD4E cells was not addressed in this study. Inhibition of HIV replication has been demonstrated by culturing purified CD8⁺ cells with infected CD4⁺ cells in transwell culture dishes that separate the two cell subsets by a semipermeable membrane (27, 28). Suppression has also been achieved by exposing infected cells to cell-free supernatants from CD8⁺ cells (2, 29). In addition, the anti-HIV effect mediated by this CD8⁺ cell antiviral factor is not HLA restricted, nor does it involve cytolysis of infected cells (14). While inhibition of HIV replication by CD8⁺ cells is thought to be mediated by a soluble cytokine, no known cytokine has been identified (15). It is not known if feline CD8⁺ cells downregulate FIV expression by direct interaction with CD4⁺ cells or via a soluble mediator. Also, it is not known if anti-FIV activity involves a lytic or nonlytic mechanism. A CD8⁺ cell-mediated cytolytic mechanism could be of particular relevance in the CD8⁺-FCD4E coculture system, in which the opportunity for development of cytotoxic CD8⁺ cells directed against FCD4E major histocompatibility complex (MHC) antigens could exist. The observation that CD8⁺ cells from normal cats do not downregulate FIV replication in FCD4E cells (Table 3) argues against development of anti-class I or anti-class II CD8⁺ cytotoxic cells. In addition, it has recently been demonstrated that ConA-IL-2-induced CD8⁺ cells from feline PBMC were highly cytotoxic for FIV-infected FCD4E cells (32) but not uninfected cells, also arguing against anti-MHC cytotoxic CD8⁺ cells. However, it is possible that the CD8⁺ LAK cells described by Zhao et al. (32) to be highly cytotoxic for FIV-infected CD4⁺ lymphocytes could be the same cells mediating the anti-FIV activity described in this study. Studies to identify the mechanism of CD8⁺ regulation of FIV replication are in progress.

It is also possible that the mechanism(s) by which CD8⁺ cells regulate FIV replication in CD4⁺ cells is not immunological in nature. FIV has been shown to infect CD8⁺ cells in vivo (5), and we have recently demonstrated by RT-PCR that CD8⁺ cells from freshly drawn blood actively synthesize FIV mRNA (unpublished data). Thus, despite the fact that CD8⁺ cells apparently are not eliminated from the circulation in FIV-infected cats (6), they could potentially have an effect on infection of CD4⁺ cells. It is possible that the CD8⁺ infection is an abortive or tolerant infection, inducing defective interfering particles that when released suppress infection of CD4⁺ cells with infectious virus. Additional studies will be required to resolve these questions.

In summary, these studies demonstrate the existence of CD8⁺ cell-mediated antiviral activity in naturally and experimentally FIV-infected cats. Similar to the CD8⁺ anti-HIV lymphocytes in HIV-infected patients, these CD8⁺ anti-FIV lymphocytes appear to suppress FIV replication in a non-MHC-restricted manner. The significance of these anti-FIV CD8⁺ cells in the immunopathogenesis of the infection and progression of the disease has yet to be determined.

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