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Proviral Burden and Infection Kinetics of Feline Immunodeficiency Virus in Lymphocyte Subsets of Blood and Lymph Node

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Feline immunodeficiency virus (FIV) is similar to human immunodeficiency virus type 1 virologically and induces a clinical syndrome in cats comparable to human immunodeficiency virus type 1 syndrome in humans. To determine the lymphoid target cells of FIV, populations of $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, and $CD21^+$ lymphocytes (B cells) were enriched to more than 96.5% purity and then analyzed for FIV provirus by semiquantitative DNA amplification. We found FIV provirus in $CD4^+$, $CD8^+$, and B lymphocytes. In cats infected for <4 months, proviral burden was greatest in $CD4^+$ cells, followed by B cells and then by $CD8^+$ cells. In cats infected for more than 5 years, proviral burden was greatest in B cells, followed by $CD4^+$ cells and then by $CD8^+$ cells. The total proviral burden was >1 $\log_{10}$ higher in acutely infected cats than in chronically infected cats, primarily because of a higher level of $CD4^+$ infection in the acutely infected cats. A comparison of proviral loads in mesenteric lymph node and peripheral blood mononuclear cells in acutely or chronically infected cats revealed no significant difference. A kinetics study of FIV infection demonstrated that all lymphocyte subpopulations were infected by 4 weeks postinoculation. Virus was isolated from $CD4^+$, $CD8^+$, and B cells in vitro, and reverse transcriptase PCR demonstrated that all subsets contained viral RNA in vivo and therefore are productive reservoirs for FIV.

Feline immunodeficiency virus (FIV) is a lentivirus associated with acquired immunodeficiency in cats and is an important animal model for human immunodeficiency virus (HIV) infection (10, 21). FIV, like HIV, induces a disease syndrome defined by three clinical stages (21, 22, 25): first, an acute flu-like illness occurring within a few weeks after infection; second, a clinically asymptomatic phase of variable duration; and, finally, immunodeficiency and associated opportunistic infections (12, 21, 22, 25). FIV and HIV are characterized by a gradual decline in $CD4^+$ T lymphocytes and increased susceptibility to an array of opportunistic pathogens (1, 14, 16). Despite the similarities in the clinical disease and pathogenesis of FIV and HIV, these viruses use different receptors and have different in vivo target cell ranges.

The CD4 molecule is the major cellular receptor for HIV, although other molecules have been implicated, particularly for the infection of cell types other than $CD4^+$ lymphocytes (6, 13, 17). It has recently been shown that expression of feline CD4 is insufficient to confer infectibility on FIV-resistant cell lines, suggesting that other molecules may be required along with CD4 for infection of feline cells or that a non-CD4 molecule is the major receptor for FIV (19). The use of a non-CD4 molecule for infection may explain FIV's broad target cell range. The cellular tropism of FIV in vivo has been reported to include $CD4^+$ and $CD8^+$ lymphocytes, macrophages, and immunoglobulin G (IgG)-positive lymphocytes (2–4, 9). The last finding suggests that B cells may be a target.

To better understand the importance of the in vivo target cell range of FIV, we sought to confirm the phenotype of lymphocyte targets, determine the kinetics of infection of lymphocyte subpopulations, and semiquantify the proviral burden within lymphocyte subpopulations. In doing so, we also compared the proviral burdens of lymphocyte subpopulations in the blood and lymph node compartments. Finally, the replication status of FIV within lymphocyte subpopulations in vivo was determined.

MATERIALS AND METHODS

Animals, inoculum, and sampling. Specific-pathogen-free cats were obtained from the breeding colony of the Feline Retrovirus Research Laboratory of the School of Veterinary Medicine, University of California, Davis, and were housed in small groups in infectious disease isolation facilities provided by Animal Resources Services. Animals sampled during the chronic and acute phases of infection were inoculated at 2 to 12 and 6 to 10 months of age, respectively. Blood from either awake or ketamine-HCl-anesthetized cats was collected by jugular venipuncture into EDTA and heparin. Mesenteric lymph nodes were harvested at necropsy.

Three groups of animals were used in these studies (Table 1). Group I animals were inoculated intraperitoneally with 1 ml of blood from a cat (no. 5000) chronically infected with FIV-Petaluma, a biologic isolate (21). For the prospective part of the study (group II), 10 adolescent cats were inoculated with FIV-Petaluma (1 ml of blood from chronically infected cat no. 5000). In this study, two cats each were sacrificed at 2, 4, 6, 8, and 13 weeks postinoculation (p.i.) and mesenteric lymph nodes were harvested. Peripheral blood mononuclear cells (PBMC) were collected from six cats at the first four time points, from four cats at the fifth time point, and from two at the final time point. In the third study (group III), mesenteric lymph nodes from five cats were harvested at necropsy, 20 weeks p.i. This group of animals was inoculated with 1,000 50% tissue culture infective doses of FIV-Petaluma grown in PBMC in vitro. The PBMC were inoculated with plasma from a chronically infected cat (no. 5027), and cultures were maintained for less than 3 days to prevent attenuation of the original inoculum. The clinical, hematological, and virological responses of all cats were similar to those previously described and are well characterized for FIV-Petaluma (2, 8, 27).

Two-color immunophenotypic labelling. Mononuclear cells collected as described below were washed twice with a solution of phosphate-buffered saline, 5% fetal calf serum, and 0.1% sodium azide (PBS-FCS-NaN_3), centrifuged at 300 × *g* for 3 min, and incubated for 20 min in the appropriate dilution of fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody specific for CD4 (Fe1.7B12; P. Moore) or CD8 (fT2; Southern Biotechnology Associates, Inc., Birmingham, Ala.) and phycoerythrin (PE)-conjugated monoclonal antibody specific for CD21 (CA2.1D6; P. Moore). The cells were then washed twice with PBS-FCS-NaN_3 and analyzed by flow cytometry by using

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TABLE 1. Treatment groups, tissues, and collection times

Group	Cat no.	Status	No. of wk p.i.[a]	Tissue(s)[b]
I	5008	Control	NA	PBMC
	33	Control	NA	LN
	16	Control	NA	LN
	18	Control	NA	LN
	5144	FIV inoculated	9.0	PBMC, LN
	5142	FIV inoculated	10.0	PBMC, LN
	5138	FIV inoculated	14.0	PBMC, LN
	5166	FIV inoculated	14.0	PBMC, LN
	2433	FIV inoculated	>104	PBMC, LN
	12	FIV inoculated	200.0	PBMC, LN
	36	FIV inoculated	253.0	PBMC, LN
	21	FIV inoculated	268.0	PBMC, LN
	22	FIV inoculated	268.0	PBMC, LN
	6	FIV inoculated	282.0	PBMC, LN
	9	FIV inoculated	282.0	PBMC, LN
	10	FIV inoculated	282.0	PBMC, LN
	2436	FIV inoculated	306.0	PBMC, LN
II	91312	FIV inoculated	Multiple	PBMC, LN
	92188	FIV inoculated	Multiple	PBMC, LN
	92074	FIV inoculated	Multiple	PBMC, LN
	92261	FIV inoculated	Multiple	PBMC, LN
	5209	FIV inoculated	Multiple	PBMC, LN
	5214	FIV inoculated	Multiple	PBMC, LN
	5208	FIV inoculated	Multiple	PBMC, LN
	5212	FIV inoculated	Multiple	PBMC, LN
	5220	FIV inoculated	Multiple	PBMC, LN
	5221	FIV inoculated	Multiple	PBMC, LN
III	5280	FIV inoculated	22	LN
	5293	FIV inoculated	22	LN
	5295	FIV inoculated	22	LN
	5296	FIV inoculated	22	LN
	5299	FIV inoculated	22	LN
	5257	Control	22	LN
	5255	Control	22	LN

[a] NA, not applicable. "Multiple" indicates samples collected at 2, 4, 6, 8, and 13 weeks p.i.
[b] LN, lymph node.

standard two-color analysis settings with a FACScan (Becton Dickinson, San Jose, Calif.).

Magnetic cell sorting. Lymph nodes were dissociated into a single-cell suspension with a Cellector (Bellco Glass Inc., Vineland, N.J.). Viable mononuclear cells were then harvested from the lymph node suspension or peripheral blood by density gradient centrifugation through Ficoll-Hypaque (density, 1.077; Sigma, St. Louis, Mo.). Cells were kept at 4°C throughout the labelling and sorting procedure. Mononuclear cells were washed twice with PBS-FCS-NaN_3, centrifuged at 300 × *g* for 3 min, and incubated for 20 min in the appropriate dilution of FITC-conjugated mouse monoclonal antibody specific for CD4 (Fe1.7B12), CD8 (fT2), or CD21 (CA2.1D6). The cells were then washed twice with PBS-FCS-NaN_3 and incubated with rat anti-mouse IgG1 microbeads (Miltenyi Biotec, Auburn, Calif.) for 15 min at 4°C. The cells were then washed twice with PBS-FSC-NaN_3. Labelled cells were harvested by magnetic cell sorting with a MiniMACS (Miltenyi Biotec) according to the manufacturer's instructions. The purity of magnetically sorted lymphocytes was verified by flow cytometry with a FACScan (Becton Dickinson); in all cases the purity was greater than 96.5%, and usually it was greater than 98.0%.

PCR and reverse transcription. The number of FIV-infected cells was determined by whole-cell, endpoint dilution, semiquantitative PCR (23). From a tube containing 1.1×10^6 mononuclear cells in 110 μl of PBS (prepared as described above), 10-fold serial dilutions (10 μl transferred) were made into tubes containing 1×10^6 PBMC in 100 μl of PBS from an uninfected, specific-pathogen-free cat. Each dilution of cells was then pelleted and lysed in 100 μl of lysing buffer (10 mM Tris-Cl [pH 8.3], 0.45% Nonidet P-40, 0.45% Tween 20, 50 μg of proteinase K per ml) and incubated for 3 h at 56°C and then for 10 min at 100°C. Thus, each lysed dilution contained DNA from 10^6 total cells, so that the concentration of DNA added to each PCR mixture was equalized. Seminested PCR was performed (on 10 μl of cell lysate from each dilution) with primers specific for the FIV Gag gene sequence (26). After two PCR rounds, each consisting of 35 cycles (1 min of template denaturation at 94°C, 1 min of primer annealing at 55°C, and 1.5 min of primer extension at 72°C), PCR products were analyzed by electrophoresis through 2% agarose–0.5× Tris-borate–EDTA buffer followed by ethidium bromide staining. The endpoint was the last dilution of cells resulting in a positive PCR signal. From this, the minimum number of cells containing proviral FIV DNA could be determined. FIV-specific PCR products of 486 bp (round 1) and 159 bp (round 2) have been confirmed by Southern blotting. We have determined the sensitivity of this seminested PCR to be between 1 and 10 FIV-infected cells per reaction and 1 to 10 FIV-containing plasmid copies.

For reverse transcription-PCR, RNA was extracted with the RNeasy total RNA kit (Qiagen, Chatsworth, Calif.). Samples were incubated with 40 U of RNase-free DNase (Boehringer Mannheim) and 20 U of RNasin (Promega Inc., Madison, Wis.) at 37°C for 60 min and then at 99°C for 5 min. The RNA concentration was determined by measuring the A_{280} (Genquant II spectrophotometer; Pharmacia, Uppsala, Sweden), and 0.4 μg of RNA was reverse transcribed with a reverse transcription kit (Promega). An FIV Gag-specific primer (NP38) was used for reverse transcription, and the reaction mixture was incubated for 1 h. Samples were then subjected to PCR with seminested Gag primers as described above. Parallel samples were processed without reverse transcriptase to ensure that no contaminating proviral DNA was present in the extracted RNA.

Virus isolation. Sorted or unsorted lymphocytes (10^6) were cocultured with PBMC from a noninfected donor cat in the presence of 5 μg of concanavalin A per ml and 100 U of human recombinant interleukin-2 per ml. Culture supernatants were monitored weekly for viral p24 by enzyme-linked immunosorbent assay as previously described (7). Cultures were considered positive when p24 was detected for 2 consecutive weeks and negative if no p24 was detected after 6 weeks in culture.

RESULTS

B-cell antibody and magnetic cell sorting. Monoclonal antibody CA2.1D6 was raised against canine lymphocytes and has been shown to be specific for CD21, a cell-surface molecule found exclusively on B cells (5, 18). Feline lymphocytes double-labelled with monoclonal antibodies Fe1.7B12-FITC and CA2.1D6-PE or fT2-FITC and CA2.1D6-PE demonstrated the cross-reactivity and specificity of CA2.1D6 to be exclusive of $CD4^+$ and $CD8^+$ lymphocytes in cats (Fig. 1). In addition, CA2.1D6 has been shown to immunoprecipitate a 145-kDa protein from radiolabelled feline lymphocytes that is comparable to the 145-kDa protein observed in dogs and to 140-kDa proteins identified as CD21 in humans and mice (data not shown). Thus, we concluded that monoclonal antibody CA2.1D6 specifically identified feline B cells.

Proviral burdens in lymphocyte subsets of acutely infected and chronically infected cats. To determine the phenotype of and semiquantify FIV-infected cells in vivo, magnetically sorted blood lymphocytes from four acutely infected (infected for less than 4 months) and nine chronically infected (infected for 24 to 77 months) cats were evaluated by semiquantitative PCR (Fig. 2). In both acutely infected and chronically infected animals, $CD4^+$, $CD8^+$, and B lymphocytes contained FIV proviral DNA (Fig. 3). In acutely infected cats, a mean of 60 of 10,000 $CD4^+$ cells contained proviral DNA, compared with 10 of 10,000 $CD8^+$ cells and 30 of 10,000 B cells. In contrast, provirus was found in means of 10 of 10,000 $CD4^+$ cells, 6 of

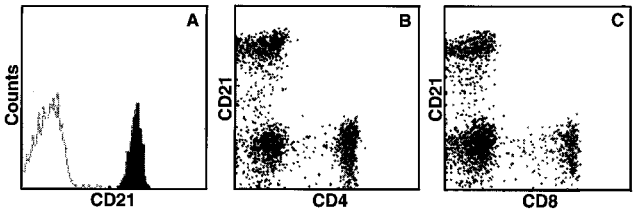

FIG. 1. Immunophenotypic labelling of feline B lymphocytes. The single-parameter histogram in panel A demonstrates the discrete labelling of the B-lymphocyte population with CD21-specific monoclonal antibody CA2.1D6. The dual-parameter histograms in panels B and C demonstrate that the lymphocyte population labelled by CA2.1D6 is mutually exclusive of the $CD4^+$ and $CD8^+$ populations, respectively.

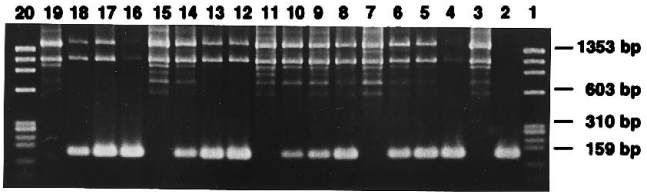

FIG. 2. Semiquantitative PCR for FIV proviral DNA in purified lymphocyte subsets. Seminested PCR was performed, and products were electrophoretically separated on a 2% agarose gel stained with ethidium bromide. Lanes 1 and 20 are ΦX174 *Hae*III markers. Lanes 2 and 3 are positive and negative controls, respectively. Lanes 4 to 19 represent whole-cell lysates of 10^5 to 10^2 lymphocytes, respectively, as follows: $CD4^+$ (lanes 4 to 7), $CD8^+$ (lanes 8 to 11), $CD21^+$ (lanes 12 to 15), and unfractionated (lanes 16 to 19). Samples are from peripheral blood lymphocytes of a single cat at 8 weeks p.i. The FIV-specific band is 159 bp.

10,000 $CD8^+$ cells, and 30 of 10,000 B cells in chronically infected cats. The mean number of provirus-containing cells in total PBMC was significantly greater ($P \leq 0.018$) in acutely infected cats (80 of 10,000 cells) than in chronically infected cats (10 of 10,000 cells).

Kinetics of proviral burden in blood and lymph node lymphocyte subsets during the acute phase of FIV infection. To determine whether all lymphocyte subsets were infected initially or whether one subset was preferentially infected and the virus subsequently spread to other subsets, a prospective study to identify FIV lymphoid target cells during the acute phase of infection (0 to 91 days p.i.) was performed. At 2 weeks p.i., FIV provirus was detected in peripheral blood $CD4^+$ and $CD8^+$ cells but not B cells. Conversely, FIV provirus was detected in lymph node $CD8^+$ and B cells but not $CD4^+$ cells. By 4 weeks p.i., peripheral blood $CD4^+$, $CD8^+$, and B cells contained high levels of proviral DNA (mean values for infected cells, 435 of 10,000, 345 of 10,000, and 62 of 10,000, respectively). Proviral levels increased to 1,000, 505, and 550 infected cells per 10,000 $CD4^+$, $CD8^+$, and B cells, respectively, at the final time point (Fig. 4A). Figure 4B shows the kinetics of lymphocyte subset infection in lymph nodes, which were similar to those observed in peripheral blood.

Comparison of proviral burdens in blood and lymph nodes in acutely infected and chronically infected cats. The proviral burden in PBMC was compared with the burden in lymph node mononuclear cells at five time points during the acute phase of infection. At no time was there any significant difference in the proviral load in these tissues. In fact, lines plotting the results are nearly superimposed (Fig. 5A). When the same comparison was made in four chronically infected cats, no difference was observed in two cats and peripheral blood had a greater proviral load than lymph nodes in the other two cats (Fig. 5B). However, for the group as a whole, no significant difference between the tissues was observed.

Virus isolation from lymphocyte subsets. Virus isolation was performed with sorted and unsorted cells from four acutely infected and nine chronically infected cats (group I). Virus was readily produced from lymphocytes of all phenotypes in acutely infected cats. Occasionally, lymphocyte cultures from chronically infected cats did not produce virus; however, purified lymphocytes of all phenotypes from the majority of cats did yield infectious particles. Thus, $CD4^+$, $CD8^+$, and B lymphocytes are all capable of producing infectious virus in vitro (data not shown).

Virus replication in lymph node lymphocyte subsets. To determine whether $CD4^+$, $CD8^+$, and B lymphocytes support viral replication in vivo, mesenteric lymph nodes were harvested from five FIV-infected cats at 20 weeks p.i. (group III) and reverse transcription-PCR was performed on sorted lymphocyte subsets. In all five cats, lymphocytes of all phenotypes contained FIV RNA, indicating active replication of FIV in vivo (Table 2).

DISCUSSION

In the investigation of FIV pathogenesis, it is important to define the spectrum of viral target cells. Target cells of HIV infection have been intensely studied (reviewed in reference 15). Comparing the host-virus interaction of HIV in humans with that of FIV in cats may help identify the important elements influencing the pathogenesis of these similar viruses. In these studies, we have confirmed the in vivo lymphocyte targets of FIV, determined the kinetics of infection of lymphocyte subpopulations, semiquantified the viral burden in lymphocyte subpopulations of acutely infected and chronically infected cats, and determined the replication status of FIV in lymphocyte subsets.

It has previously been reported that FIV infects $CD4^+$ and $CD8^+$ lymphocytes and macrophages in vivo and in vitro (2–4, 9). In addition, English et al. have reported that IgG^+ cells are infected by FIV, suggesting that B lymphocytes are an in vivo target (9). Although the data were compelling, the lack of a B-cell-specific antibody precluded definitive conclusions. Using a monoclonal antibody directed against canine CD21 and cross-reacting with feline CD21, we have shown here that B cells are indeed an in vivo target population for FIV. B-cell infection is unique to FIV among the immunodeficiency-inducing lentiviruses, since careful evaluation of B cells from HIV-infected humans and simian immunodeficiency virus (SIV)-infected monkeys has not revealed proviral DNA (7a, 11, 24). The divergence of FIV from SIV and HIV in lymphocyte target population but its similarity in pathogenesis reemphasizes the association of immunodeficiency-inducing lentiviruses with the ability to infect $CD4^+$ lymphocytes.

Like previous investigators, we observed that provirus levels were higher in $CD4^+$ cells during the acute phase of infection, while B cells contained the majority of provirus during the chronic phase (9). We conclude that this shift was due to a decline in provirus in the $CD4^+$ population and was reflected as a decline in total proviral burden in cats chronically infected with FIV compared with that in acutely infected cats. This is in contrast to the data of English et al., which suggested that the proviral burden increased in B cells during the chronic phase of infection (9). This discrepancy may be a result of the method of defining the B-cell population or of the technique for com-

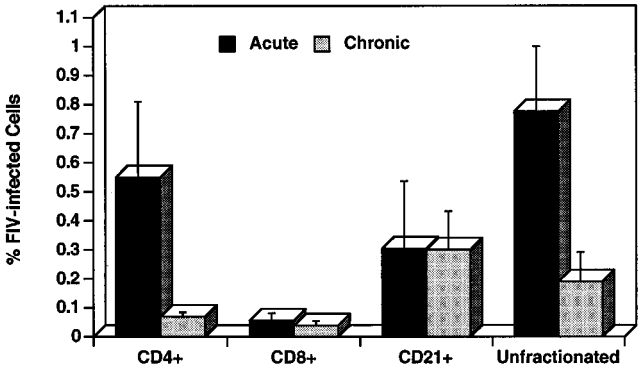

FIG. 3. Comparison of FIV proviral loads in lymph node lymphocyte subsets of acutely infected ($n = 4$) and chronically infected ($n = 9$) cats. Error bars represent the standard errors of the means.

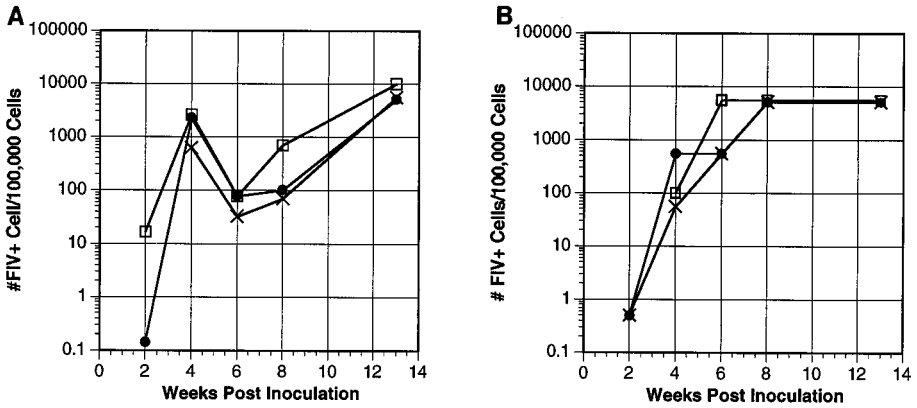

FIG. 4. Kinetics of FIV infection of lymphocyte subsets in peripheral blood (A) and mesenteric lymph nodes (B). Four samples were used for the 2-, 4-, 6-, and 8-week time points, and two samples were used at the final time point for peripheral blood lymphocytes. B and $CD4^+$ cells were negative by PCR at 2 weeks p.i. in peripheral blood and lymph nodes, respectively. Two samples were used for lymph node subsets at all time points. Data are expressed as means of samples. Symbols: □, $CD4^+$ cells; ●, $CD8^+$ cells; ×, B cells.

parison of proviral burdens. In these studies we defined B cells by the expression of CD21 and determined the number of cells containing provirus, while English et al. defined B cells by the presence of surface IgG and determined the amount of provirus relative to cellular DNA. The significant decline in the percentage of infected $CD4^+$ cells between the acute and chronic phases of infection and the lack of such changes in the $CD8^+$-cell and B-cell populations again suggest a unique interaction of FIV with $CD4^+$ cells. Possible explanations for the decline in the percentage of infected $CD4^+$ cells include elimination of a $CD4^+$ subset, targeting of the immune response to infected $CD4^+$ cells, or altered $CD4^+$ cell turnover kinetics.

Studies of lymphocyte subset infection kinetics in vivo demonstrated that $CD4^+$, $CD8^+$, and B cells are infected to high levels within 4 weeks of inoculation. This suggests that lymphocytes of all phenotypes are infected by a similar mechanism. In addition, since the virus receptor is not the CD4 molecule (19), it is likely that the cellular FIV receptor is an antigen common to lymphocytes of many phenotypes. Taken together, the broad target cell range and the probable use of a non-CD4 receptor raise questions about the significance of the CD4 molecule in the immunopathogenesis of FIV.

The proviral burden of FIV, unlike that of HIV, was similar in lymph nodes and PBMC, thus revealing no sequestration of virus-infected cells in tissues (20). This is advantageous in that, clinically or experimentally, the proviral burden can easily be determined by sampling PBMC. The broad tropism of FIV and the shifting proviral levels within $CD4^+$ cells, however, may make evaluation of $CD4^+$ cells alone more relevant in some experiments.

Importantly, studies presented here demonstrate that $CD8^+$ and B cells do not represent a dead-end infection. Not only can lymphocytes of all the phenotypes investigated here produce infectious virus in vitro, but these cells are also actively replicating virus in vivo. There are no data to suggest that non-CD4-bearing cells are adversely affected by FIV infection, but this has not been closely investigated. Regardless, it is likely

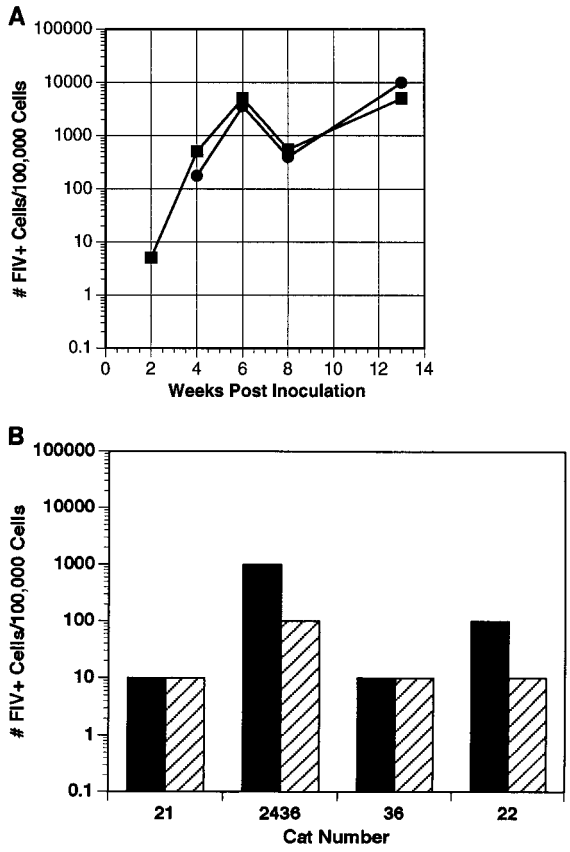

FIG. 5. Comparison of FIV proviral loads in peripheral blood and lymph nodes of acutely infected (A) and chronically infected (B) cats. (A) ■, lymph node; ●, PBMC. (B) ■, PBMC; ▨, lymph node.

TABLE 2. FIV status of mesenteric lymph node lymphocyte subsets

Cat no.	Status	FIV status of subset[a]			
		$CD4^+$	$CD8^+$	$CD21^+$	Unfractionated
5280	Inoculated	+	+	+	+
5293	Inoculated	+	+	+	+
5295	Inoculated	+	+	+	+
5296	Inoculated	+	+	+	+
5299	Inoculated	+	+	+	+
5257	Control	−	−	−	−
5255	Control	−	−	−	−

[a] Determined by reverse transcription-PCR.

that these populations contribute to pathogenesis at least, by maintaining a higher replicating proviral load.

In conclusion, although FIV has a much broader cell tropism, the disease caused by FIV in domestic cats is identical in many aspects to the disease caused by HIV in humans. The difference in tropism is most likely due to the use of a broadly distributed cellular receptor by FIV rather than the narrowly distributed CD4 molecule used by HIV and SIV. This finding has two implications: (i) the important issue in lentivirus-induced immunodeficiency may be not the receptor by which the virus enters into T cells but its cytopathic effect on the $CD4^+$ T cells, and (ii) the differentiating feature between the immunodeficiency-causing lentiviruses (FIV, HIV, and SIV) and non-immunodeficiency-causing lentiviruses may be the ability of the former subgroup to infect $CD4^+$ T cells. Although it may appear at first that differences in the genomic makeup and cell tropism of FIV and HIV negate FIV as a model for lentivirus-induced immunodeficiency in humans, it can be argued that the differences may provide more important clues than the similarities as to how lentiviruses induce immunodeficiency.

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