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Puma Lentivirus Is Controlled in Domestic Cats after Mucosal Exposure in the Absence of Conventional Indicators of Immunity

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A high percentage of free-ranging pumas (*Felis concolor*) are infected with feline lentiviruses (puma lentivirus, feline immunodeficiency virus Pco [FIV-Pco], referred to here as PLV) without evidence of disease. PLV establishes productive infection in domestic cats following parenteral exposure but, in contrast to domestic cat FIV, it does not cause T-cell dysregulation. Here we report that cats exposed to PLV oro-nasally became infected yet rapidly cleared peripheral blood mononuclear cell (PBMC) proviral load in the absence of a correlative specific immune response. Two groups of four specific-pathogen-free cats were exposed to PLV via the mucosal (oro-nasal) or parenteral (i.v.) route. All animals were PBMC culture positive and PCR positive within 3 weeks postinfection and seroconverted without exhibiting clinical disease; however, three or four oro-nasally infected animals cleared circulating proviral DNA within 3 months. Antibody titers reached higher levels in animals that remained persistently infected. PLV antigen-induced proliferation was slightly greater in mucosally inoculated animals, but no differences were noted in cytotoxic T-lymphocyte responses or cytokine profiles between groups. The distribution of virus was predominantly gastrointestinal as opposed to lymphoid in all animals in which virus was detected at necropsy. Possible mechanisms for viral clearance include differences in viral fitness required for crossing mucosal surfaces, a threshold dose requirement for persistence, or an undetected sterilizing host immune response. This is the first report of control of a productive feline or primate lentivirus infection in postnatally exposed, seropositive animals. Mechanisms underlying this observation will provide clues to containment of immunodeficiency disease and could prompt reexamination of vaccine-induced immunity against human immunodeficiency virus and other lentiviruses.

Human immunodeficiency virus (HIV)-induced AIDS is an emergent disease of humans capable of causing progressive fatal immunological impairment. HIV arose from human contact with nonhuman primates infected with simian immunodeficiency virus (SIV). In contrast to HIV, SIVs originating in African primate hosts are thought to be relatively ancient viruses which result in apathogenic infections in their native hosts. Typically, 50% of adults in a given population are infected with SIV in the greater than 30 species of African primates that are reported to be SIV positive (25).

Feline immunodeficiency virus (FIV) of domestic cats is analogous to HIV with respect to its ability to infect lymphocytes, cause fatal immune dysfunction, and its relatively recent detection in the domestic cat population. FIV-induced immune dysfunction is characterized by a progressive depletion of $CD4^+$ lymphocytes (2), mirroring HIV-induced AIDS (reviewed in references 32, 36). FIV infection in domestic cats is a small animal model which has provided insight into lentiviral maternal-fetal and transmucosal transmission, vaccine development, and pathogenesis (7, 21, 52).

Serologic surveys of 36 nondomestic feline species have revealed a minimum of 20 species which have antibodies that cross-react with FIV antigens (4). Genetic characterization of several of these viruses has determined that they are distinct from each other and related to domestic cat FIV (5). The clinical consequences of infection with the lentiviruses indigenous to wild and captive nondomestic felid populations have not been well studied; however, as with SIV infection of sooty mangabeys or African green monkeys, it appears that these infections are relatively avirulent (11). Sequence comparison of the *pol* and *env* genes from domestic and nondomestic FIVs demonstrate a high degree of divergence in puma lentiviruses (PLVs) relative to domestic cat isolates, suggesting that PLV has been in puma lineages longer than FIV has been in domestic cat populations. PLV has high seroprevalence in native populations, particularly in aged animals, suggesting that the virus is both highly infectious and relatively nonpathogenic (8, 13, 16). It has been postulated that lentiviral-host adaptation has occurred over time in pumas infected with PLV, either in terms of immune response and/or viral evolutionary selection, resulting in relative apathogenic infection. Other evidence for host-viral adaptation in PLV which parallels SIV infection of African primates is the presence of a lower rate of nonsynonymous compared to synonymous genetic changes in PLV *env* (8, 13, 16). Thus, the relationship between the nondomestic cat and the domestic cat lentiviruses appears to mirror that between SIV and HIV-1, with the nondomestic cat lentiviruses and SIV being more ancient and host adapted and FIV and HIV representing more recent lentivirus introductions into a host species.

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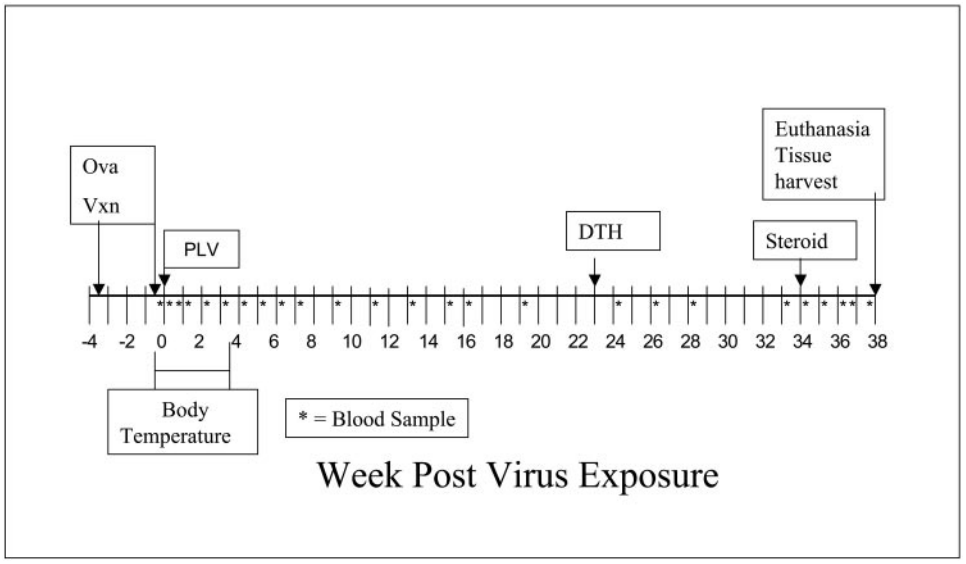

FIG. 1. Timeline of study events. Blood samples were taken very frequently early postinoculation to document initial infection, seroconversion, and cytokine profiles. Once infection was established sampling frequency was decreased, since parameters being monitored attained steady state.

Our previous studies have shown that certain isolates of PLV and lion lentivirus, (FIV-Ple, referred to here as LLV) can replicate in domestic cat cells and infect domestic cats, although no disease results (48, 51). Additionally, infection of domestic cats with PLV or LLV evokes strong antilentiviral responses apparently capable of restricting subsequent infection with virulent FIV (50, 51). The following study was conducted to determine if PLV could infect domestic cats via the mucosal route in order to simulate a natural infection of a susceptible host with an avirulent lentivirus. Subsequent evaluations of viral kinetics and host immune responses were made to compare this route with parenteral inoculation and provide correlates with virus control by the host.

MATERIALS AND METHODS

Animals. Ten 8-week-old specific-pathogen-free (SPF) cats from two litters were purchased from Cedar River Laboratory (Mason City, Iowa). Animals were randomized by litter and sex and were housed in groups of two (control) or four (experimental infection) in isolation rooms in an AAALAC-accredited animal facility. All procedures were approved by the CSU Institutional Animal Care and Use Committee prior to initiation. Figure 1 summarizes the sequence of study events.

Vaccination with ovalbumin. Each animal received 50 to 100 μg of ovalbumin (Sigma, St. Louis, Mo.) with the RIBI adjuvant system (Corixa, Seattle, Wash.) as adjuvant via the subcutaneous route 25 and 5 days prior to exposure to tissue culture medium or PLV.

Virus. PLV-1695 was isolated from a Vancouver Island puma and was passaged once in freshly isolated domestic cat peripheral blood mononuclear cells (PBMCs) (49) and then four times in the feline lymphoblastoid cell line MYA-1 (37). The cell-free culture supernatant used as inoculum had a titer of $10^{4.7}$ 50% tissue culture infective doses/ml on MYA-1 cells and 5 million copies/ml by real-time PCR.

Experimental inoculation. Animals were lightly anesthetized with intravenous (i.v.) ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa) at a dosage of 0.1 mg/kg of body weight with 0.1-mg/ml Acepromazine (Vedco, St. Joseph, Mo.) and received either 1 ml of cell-free tissue culture supernatant dropped into the nose and mouth using a micropipette or 0.8 ml i.v.

Clinical observations. Animals were observed for clinical signs at least daily throughout the study. Body temperature was measured every other day from 2 days prior to until 22 days after exposure to virus. Lymph nodes were palpated at these time points. Clinical examinations were also made at these times and then weekly until 16 weeks post-virus exposure. Body weights were measured weekly from 4 weeks prior to until 12 weeks post-virus exposure. Clinical monitoring was not measured intensively after this time, as all cats were consistently clinically normal. Blood samples were obtained by venipuncture of the jugular or cephalic vein on study days (relative to virus exposure) −1, 3, 7, 10, 15, 22, 29, 35, 42, 49, 64, 79, 91, 107, 114, 135, 171, 184, 198, 233, 240, 247, 254, and 259.

Quantitation of proviral load. PBMCs were purified from heparinized whole blood using a Histopaque (Sigma) gradient according to the product insert. DNA was extracted from 1 million PBMCs using the Qiamp blood mini DNA kit (QIAGEN, Valencia, Calif.). DNA was eluted with 50 to 200 μl of buffer, and PLV in 5 μl was quantitated in triplicate using real-time PCR with plasmid encoded PLV as a standard. Primers and probe were designed for the *pol* region of PLV-1695. This assay has a less than 3% within and between sample variance and is sensitive to a minimum of 10 copies. The difference in amplification efficiency for plasmid versus sample DNA was less than 1% (K. S. Sondgeroth, C. Leutenegger, and S. VandeWoude, submitted for publication).

Cocultivation. One million PBMCs (isolated as above) were added to 5 million MYA-1 cells in RPMI 1640 (Invitrogen Life Sciences, Carlsbad, Calif.) containing 20% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), 1% Glutamax I, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5×10^{-5} M β-2-mercaptoethanol, 100 U of penicillin/ml, 100 μg of streptomycin/ml, interleukin-2 at 100 U/ml (all from Invitrogen Life Sciences), and 9 g of glucose (Sigma)/liter. Samples were taken weekly from these cultures for at least 4 weeks and were tested for the presence of reverse transcriptase in a microplate assay. Briefly, 15 μl of culture supernatant in triplicate was incubated with 50 μl of 0.05 M Tris (pH 7.8) with 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 2 mM dithiothreitol, 5 nM oligo(dT), 0.05% NP-40, poly(A) at 50 μg/ml, and ^{32}P at 20 μCi/ml for 90 to 120 min at 37°C. Aliquots of 2.5 μl of each reaction mixture were spotted onto a nylon filter (Wallac, Turku, Finland) and allowed to dry. Unincorporated label was washed away with five 10- to 60-min washes with 0.03 M sodium citrate, pH 7.0, in 0.3 M sodium chloride (SSC) buffer, and the membrane was then fixed in 100% ethanol. Counts per minute were measured using a Microbeta Counter (Wallac).

Cytokines. Cytokines were quantitated by real-time PCR using the method of Leutenegger et al. (31). Five to 10 million PBMCs (purified as above) were dissolved in TRIzol (Sigma) at 10 million cells/ml. cDNA was reverse transcribed from RNA which had been purified by extracted phenol-chloroform extraction and ethanol precipitation. Cytokine expression levels for IL-10, IL12p40, and interferon gamma were quantitated relative to that of the cellular housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the formula $2^{-\Delta CT}$, where ΔCT represents the cycle at which threshold is reached for the GAPDH is subtracted from the cycle at which threshold is reached for the cytokine.

Hematology. Total white and red blood cell counts were measured using a Coulter Z1 (Coulter, Miami, Fla.). Differential counts were performed manually, and the percentages of lymphocytes positive for CD4 and CD8 were analyzed by flow cytometry. Monoclonal antibodies to feline CD4 and CD8 were obtained from Southern Biotechnology Associates (Birmingham, Ala.). Two $\times 10^5$ to 5 $\times 10^5$ PBMCs were incubated for 20 to 60 min at room temperature in monoclonal antibody at 5 μg/ml in flow buffer (PBS containing 2% fetal bovine serum and 0.2% sodium azide). Cells were then washed twice in flow buffer, resuspended in 100 μl of fluorescein-labeled sheep anti-mouse IgG (Sigma) at 10 μg/ml in flow buffer, and incubated for 20 to 60 min at room temperature in the dark. Cells were washed once in flow buffer and then analyzed with a Coulter EPICS XL MCL flow cytometer (Beckman Coulter, Miami, Fla.). List mode files were analyzed using FlowJo (Tree Star Inc., San Carlos, Calif.). Total cell counts for each phenotype were calculated by multiplying the total white blood cell count by the percentage of lymphocytes in the sample as determined by the differential count and then by the percentage of lymphocytes expressing that phenotype.

VN titer. Virus-neutralizing antibody (VN) titer was determined by adding a constant amount of virus to serial twofold dilutions of plasma, starting at a 1:5 final dilution. Following a 90-min incubation at 37°C with 5% CO_2, 100 μl of the virus-plasma mixture was added in triplicate to MYA-1 cells seeded at 10^5 cells/well in 96-well plates. The virus inoculum was titrated in triplicate using 10-fold serial dilutions. Virus was detected by reverse transcriptase assay after 14 days incubation at 37°C with 5% CO_2, and titers of virus inoculum and antibody were calculated using the Spearman-Kärber method (27). The virus challenge for the VN assay was 75 virus particles per well.

Polyacrylamide gel electrophoresis and Western blotting. Four to 10 μg of PLV which had been purified by centrifuging cell-free culture supernatant through 20% sucrose for 2 h at 100,000 $\times$ *g* using a Beckman L-70 ultracentrifuge (Beckman, Fullerton, CA) was separated on a 4 to 15% polyacrylamide gel using the method of Laemmli (30). Separated proteins were transferred to polyvinyl difluoride membrane, which was then blocked in 5% nonfat dry milk diluted in PBS. Blots were then stored at −20°C until used. Blot strips were incubated for 1 h with plasma diluted 1:50 in PBS and then washed with three changes of PBS containing 0.05% Tween 20 (PBS/Tween). Alkaline phosphatase-labeled goat anti-cat IgG, gamma chain specific, was added at 0.05 μg/ml and the blot was incubated for 1 h. After three washes with PBS/Tween, phosphatase substrate (BCIP/NPT; Kirkegaard & Perry, Gaithersburg, Md.) was added and blots were incubated until the color was developed. Analysis was confined to examination of the response to Gag protein, as Env is typically more difficult to detect, possibly due to loss of Env epitopes during the purification process.

CTL activity. Cytotoxic T-lymphocyte (CTL) activity was measured by flow cytometry using a method modified from that of Fischer et al. (23). Freshly harvested effector PBMCs were incubated for 4 h at ratios of 100:1, 30:1, and 10:1 with autologous PBMCs (targets) which had been cultured in the presence of IL-2 and concanavalin A (ConA) for 7 days and which had been superinfected with PLV 3 to 5 days before the day of assay. Prior to the coincubation of targets and effectors, target cells were banded in Histopaque (Sigma) to remove dead cells and were then labeled with the lipophilic dye PKH (Sigma). After the 4-h coincubation, propidium iodide (PI) was added to each well. Cells were then analyzed with a Coulter EPICS XL MCL flow cytometer (Beckman Coulter). List mode files were analyzed using FlowJo (Tree Star Inc.). Percent lysis was calculated as the percentage of PKH-labeled cells which also were stained by PI. Percent specific lysis was calculated by subtracting the percent spontaneous lysis from the percent total lysis. Maitotoxin (22) was used as a positive control for total lysis.

Lymphocyte blastogenesis. A total of 0.1 million PBMCs were incubated in triplicate with medium alone, 10 μg of PLV inactivated using AT-2 as described in reference 45, 10 or 100 μg of ovalbumin, or 3 μg of ConA for 4 days. ^3H at 1 μCi/well was then added, and the cells were harvested onto filter paper using a 96-well cell harvester (Wallac) 20 h later. Stimulation indices were calculated by dividing the count for each sample by the count with medium alone.

Delayed-type hypersensitivity (DTH) testing. At 23 weeks post-virus exposure, inoculation sites were shaved and each animal received three intradermal injections in the mid-scapular region of saline only or 10 μg of either inactivated PLV or ovalbumin. Sites were examined for induration and redness daily for 3 days following inoculation.

Steroid-induced immunosuppression. At week 34 post-virus exposure, each animal in the experimental groups received prednisone orally at 5 mg/kg daily for 5 days. Blood samples were obtained 3 days prior to and on 5, 12, 19, and 26 days postinitiation of treatment for complete blood count and viral quantitation using real-time PCR.

Tissue localization. At 38 weeks post-virus inoculation, animals were euthanized with an overdose of barbiturate under ketamine anesthesia. Tissue samples were collected and stored frozen until assayed. Quantitation of tissue PLV was by real-time PCR using a plasmid-derived standard curve after extraction of DNA using the DNeasy tissue kit (QIAGEN) from 10 mg (spleen) or 25 mg (kidney, lung, tonsil, mesenteric lymph node, Peyer's patch, stomach, and jejunum) of tissue or 10^6 mononuclear cells (bone marrow). Methods for real-time PCR were identical to methods described for proviral load quantitation. Tissue samples obtained from FIV-infected cats were used as negative controls; provirus was never amplified from these samples.

Statistical analysis. Repeated-measures analysis of variance was used to determine if there were significant ($P < 0.05$) effects of time or group. If a significant difference was detected, individual time points were compared using the Student *t* test or Student *t* test for paired data as appropriate or Fisher's exact test for frequencies. All analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond, Wash.).

RESULTS

Clinical signs. In order to confirm the lack of FIV-like clinical signs following PLV inoculation, body temperatures were measured during the first 3 weeks postinoculation, weights were measured weekly for 12 weeks postinoculation, animals were closely observed, and lymph nodes were palpated. A transient peripheral lymphadenopathy was noted in the animals which received PLV by the i.v. route beginning on day 3 after inoculation in one animal and was evident in all animals in this group by day 6 after inoculation. Lymphadenopathy was first detected in one animal in the group which received virus by the mucosal route at day 6 after virus instillation and was present in all animals in this group by day 9 after virus instillation. Lymph nodes were no longer palpable by 10 weeks after virus exposure. There was no increase in body temperature during the 3-week period of monitoring. Although body temperatures were not measured later in the study, no lethargy, anorexia, or other signs of fever were noted during daily observation. The rate of weight gain in infected animals was slightly but not significantly ($P = 0.094$) lower than that of controls for the first 2 weeks after infection. Thus, it appears that neither mucosal nor i.v. inoculation of PLV results in illness like that of the acute phase of FIV infection.

Hematological parameters. A decline in the $CD4^+$ T-lymphocyte count is a hallmark of the immunodeficiency disease caused by FIV, HIV, and SIV. Analysis of variance revealed no effect of treatment on CD4 or CD8 count, but there was an overall effect of time; thus, each group was compared to its own preinoculation sample. As shown in Fig. 2 (top), the control group exhibited a significant increase in $CD4^+$ cell count on days 10 and 29 after virus exposure. CD4-positive cell numbers declined slightly in the group which received virus by the i.v. route on days 7 and 15 post-virus exposure ($P < 0.05$). One animal in this group had a $CD4^+$ cell count less than 200 cells/μl on days 7 and 10 post-virus inoculation. The $CD4^+$ cell count was significantly increased ($P < 0.05$) over day zero on days 35 and 91 post-virus inoculation in the i.v.-inoculated group. This may have reflected a rebound in $CD4^+$ cell numbers or may be part of the normal age-related variation; the control group also showed a statistically significant increase in $CD4^+$ cell numbers relative to baseline at different time points throughout the study. There was no statistically significant change in $CD4^+$ cell count over time in the mucosally inoculated group. $CD8^+$ cell numbers (Fig. 2, bottom) were unchanged over time in the i.v.-inoculated group and were significantly different from that on day zero in the mucosally

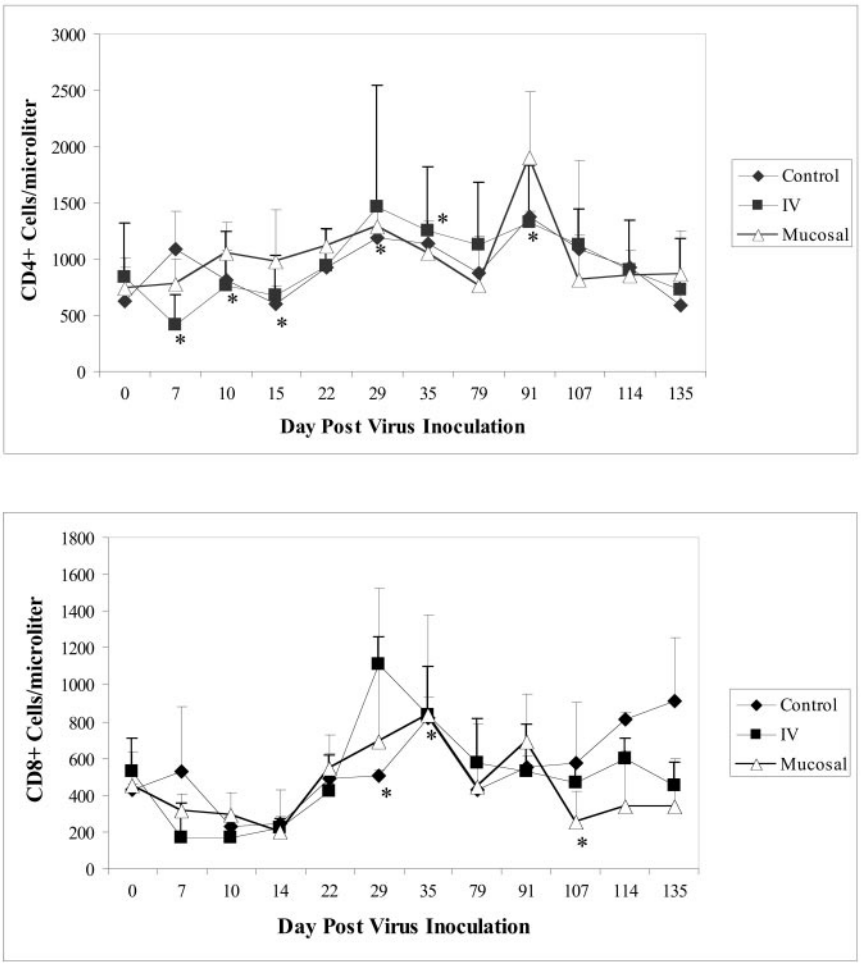

FIG. 2. Lymphocyte subsets are not altered during PLV infection. (Top) Total $CD4^+$ count. Lymphocyte numbers significantly declined only transiently after inoculation with PLV by the i.v. route. There was no decline in $CD4^+$ lymphocytes in the mucosally inoculated group. (Bottom) Total $CD8^+$ count. Lymphocyte numbers did not change in the i.v.-inoculated group, but in the mucosally inoculated group they were significantly increased on day 35 and decreased on day 107. Symbols represent the means of four animals. Bars represent 1 standard deviation. Asterisks indicate statistically significant changes ($P < 0.05$) from preexposure samples.

inoculated group on days 35 and 107 post-virus instillation ($P < 0.05$). $CD8^+$ cell count in the control kittens was significantly different from day zero on day 29.

In summary, although slight changes were observed at some time points, PLV inoculation did not induce a sustained significant decline in $CD4^+$ T lymphocyte cell count or a sustained increase in $CD8^+$ T lymphocyte counts as reported for pathogenic infections with feline or primate immunodeficiency viruses.

PBMC proviral load. Real-time PCR sensitive to 10 copies was used to detect and quantitate viral infection in PBMCs over time. Provirus was detected by day 7 in half of the animals which were inoculated with PLV by the i.v. route (Fig. 3A) and no animals in the mucosally inoculated group (Fig. 3B). At day 15 post-virus exposure, provirus was detected in PBMCs from all exposed animals with the exception of one of those mucosally inoculated. All animals exposed to virus had detectable provirus in PBMCs by day 22 after virus exposure; however, the number of copies per million PBMCs was significantly lower in the group which received virus by the mucosal route. Proviral load in animals infected by the i.v. route peaked at about 0.1 copy/cell on day 35 and then gradually declined over a period of 6 weeks to a stable level of about 0.001copies/cell. The viral kinetics in one of the mucosally inoculated animals followed a pattern similar to that of the i.v.-inoculated kittens. However, in three of four animals which received the virus by a mucosal route, proviral load dropped below the detection limit for the assay at several time points and appeared to have effectively been cleared by 11 weeks postinoculation. These data were corroborated by the absence of virus in the supernatant of cocultures of PBMCs with MYA-1 cells (data not shown; Sondgeroth et al., submitted).

Real-time PCRs using 10-fold as much DNA from the three animals which appeared to have cleared virus were set up at three separate time points (weeks 6, 26, and 34). No provirus was detected in any of these samples.

Thus, in summary i.v.-inoculated kittens developed high proviral loads rapidly, which gradually declined to a steady-state level. This pattern was repeated in one of the four mucosally inoculated cats. Initial proviral burdens in the remaining three

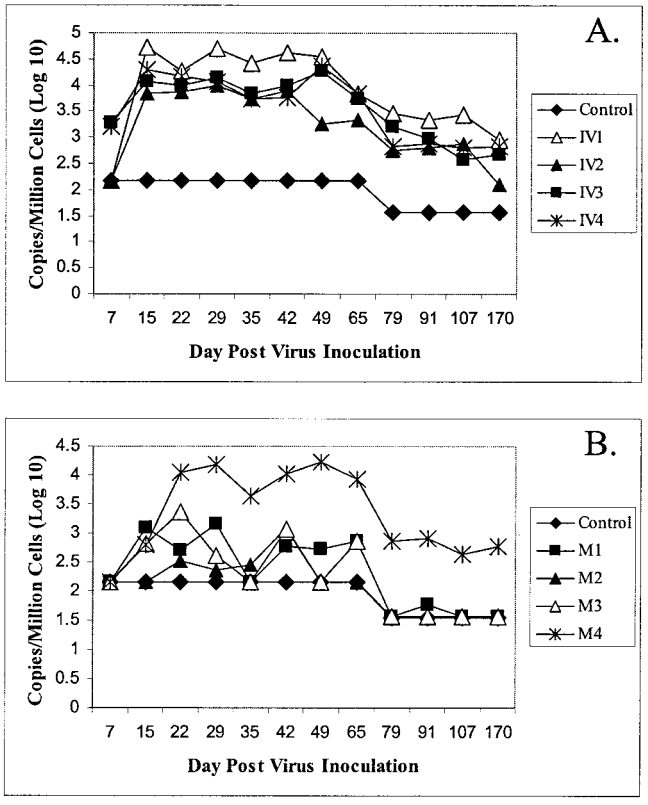

FIG. 3. Proviral load is dependent upon route of infection and was cleared following mucosal infection in three of four animals. Initial proviral load was lower in three of four mucosally inoculated cats (B) than in i.v.-inoculated cats (A). Provirus subsequently dropped below the limit of detection in these three animals. No virus was detected in nonexposed control animals. The control line indicates the lower limit of detection for the assay. This lower threshold decreased after day 65 due to a decrease in DNA elution volume and consequent enhanced assay sensitivity.

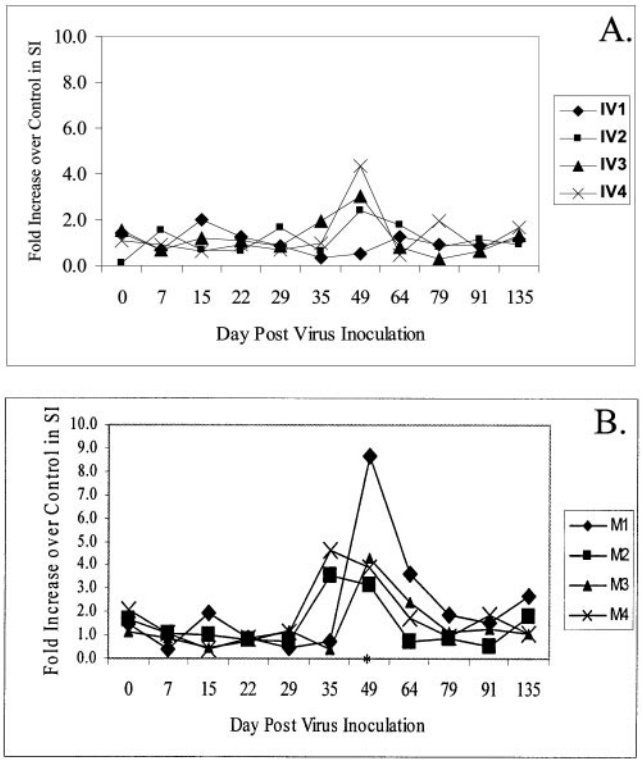

FIG. 4. PLV-specific antigen proliferation does not correlate with proviral load. Proliferation was only significantly elevated at day 49 (asterisk) post-virus exposure in the mucosally inoculated group. Individual responses are shown over time in the i.v.-inoculated (A) and mucosally inoculated (B) groups.

mucosally inoculated cats were at least 10-fold lower initially and became undetectable within 3 months of inoculation.

Serological response. In order to document seroconversion and attempt to correlate antibody response with viral clearance, plasma samples were tested by Western blotting and by serum neutralizing assays. Plasma was seropositive for Gag antigens by Western blotting in one of four of the animals infected by the i.v. route at day 15, in two of four at day 22, and in four of four at day 29 post-virus exposure. Antibody was not detected in the mucosally inoculated group until day 29 post exposure, at which point three of four were positive. The remaining animal in this group became antibody positive at day 35 postexposure. Virus-neutralizing antibody was not detected in samples from any kitten on day 15. At day 49, one animal in the i.v.-inoculated group had a VN titer of 7, and at day 107 this animal and one other in the i.v.-inoculated group had VN titers of 7 and 9, respectively. The i.v.-inoculated group developed earlier and more robust antibody responses which correlated positively with intensity of infection rather than clearance of virus.

Cell-mediated immunity. In order to examine function of PBMCs, lymphocyte proliferation assays using a nonspecific mitogen, an irrelevant antigen (ovalbumin) and PLV were conducted. Lymphocyte proliferative response to inactivated PLV antigen is shown in Fig. 4. Both groups showed a response above that of controls at weeks 5, 7, and 8 post-virus inoculation, but only the difference between the mucosally inoculated animals and the controls at week 7 was statistically significant ($P < 0.05$). A CTL response above that of controls was not observed in either infected group (data not shown). The highest specific lysis detected in infected animals was 26% and that of one of the controls was 18%, while lysis with the positive control, the cell membrane pore-forming agent maitotoxin (22), was 80%. The ability of PBMCs to proliferate in response to the mitogen ConA and to ovalbumin, with which the animals had been vaccinated prior to virus exposure, was also tested at the time points that PLV stimulation was measured (data not shown). A lower proliferative response to ConA was noted on days 7, 29, and 42 in the i.v.-inoculated group and on day 29 in the mucosally inoculated group ($P < 0.05$) relative to controls. However, there was no difference in proliferative response to ovalbumin at any time point.

These data did not detect a strong cytolytic or other cell-mediated response coincident with reduction of proviral load. A transient deficit in mitogen-induced proliferation was noted in i.v.-exposed animals early during infection, but proliferation to an irrelevant antigen did not reveal overt immunodeficiency during PLV infection.

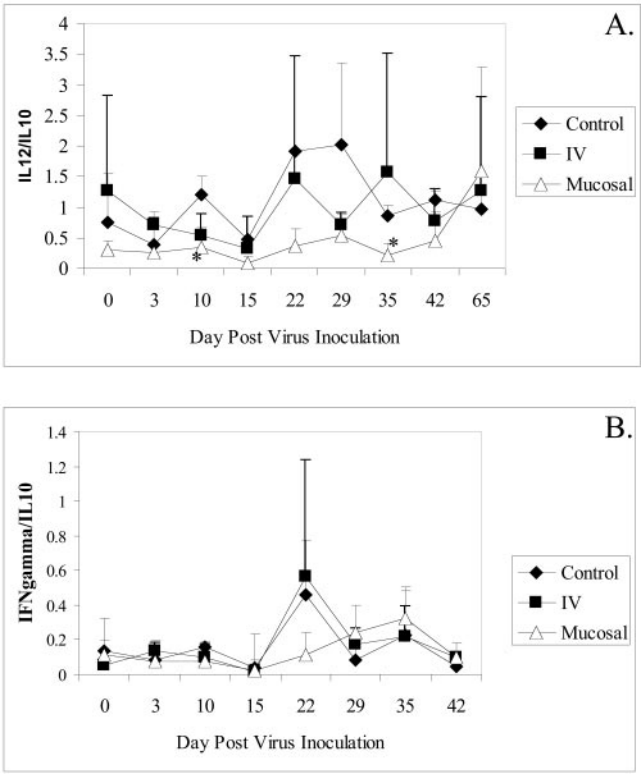

FIG. 5. Cytokine profiles are highly variable following PLV infection and do not demonstrate a significant shift toward a TH-2 response. (A) IL-12/IL-10 ratio over time. (B) IFN-γ/IL-10 ratio. Symbols represent the mean values, and bars represent 1 standard deviation. Asterisks indicate significant ($P < 0.05$) differences from control group.

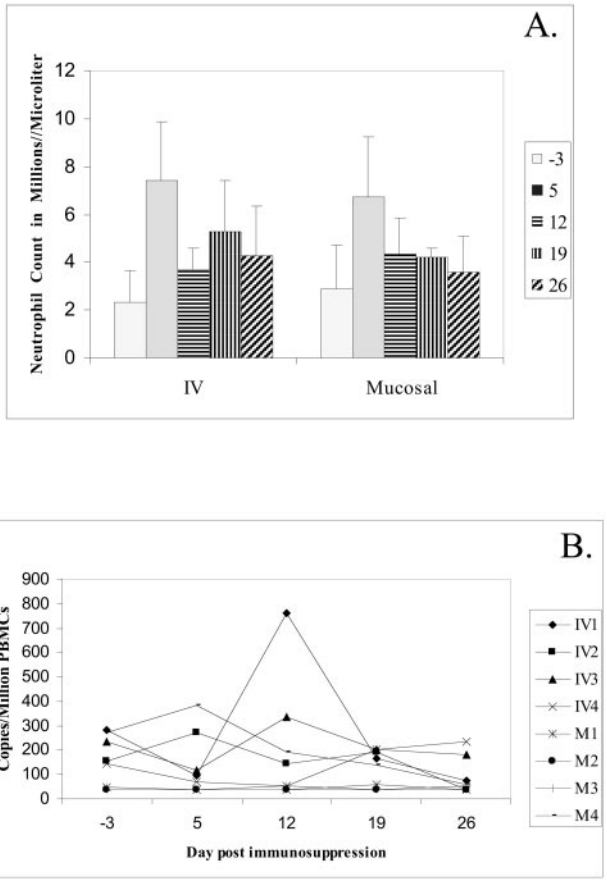

FIG. 6. Steroid treatment does not result in increased PBMC proviral burden. Eight animals which had received PLV by either a mucosal (M1 to M4) or a parenteral (IV1 to IV4) route were treated with prednisone for 5 days. Blood samples were obtained 3 days prior to prednisone treatment and on days 5, 12, 19, and 26 after treatment was withdrawn. (A) Mean neutrophil counts indicated a typical steroid-induced neutrophilia occurred. Lines above the bar represent 1 standard deviation. (B) PBMC proviral load in individual animals. Virus was below the level of detection in three animals (M1, M2, and M3) before and after steroid treatment. Of the remaining five animals (IV1 to IV4 and M4), proviral load decreased slightly in three animals and increased slightly in two animals during steroid treatment. Signal was not detected in control animals.

Cytokine expression. Since a TH-1 response has been reported to be necessary for viral clearance, we examined interleukin 12 (IL-12), IL-10, and interferon gamma (IFN-γ) levels early after infection as representative cytokines of TH-1 and TH-2 responses. Cytokine expression levels varied widely between individuals. Figure 5A shows the ratio of expression of IL-12 to that of IL-10 at several time points early in the course of infection. Ratios in the control group varied over time, with the mean ranging from 0.5 to 2. The ratios in the i.v.-inoculated group showed similar fluctuations and were not significantly different than controls. Mean ratios in the mucosally inoculated group remained below 0.5 and were significantly different than controls on days 10 and 35 post-virus inoculation. Figure 5B depicts the ratio of IFN-γ to IL-10 at these same time points. Although the i.v.-inoculated group had a higher mean IFN-γ/IL-10 ratio early after infection, this difference was not statistically significant. All mean ratios were below 1.0. No specific PBMC cytokine response was associated with clearance of virus.

DTH. As an additional measure of a cell-mediated immune response, the DTH response was tested at week 23 post-virus exposure and was negative in all kittens for both ovalbumin and PLV.

Steroid-induced immunosuppression. In an attempt to ascertain if the undetectable levels of provirus in the mucosally inoculated group were due to an immune response not detected by our assay methods, an immunosuppression regimen was initiated 33 weeks post-virus exposure. At this point, provirus was not detectable in three of four mucosally inoculated animals and had declined to less than 300 copies/million cells in i.v.-inoculated animals and the remaining mucosally inoculated animal. Although signs that the steroid was effective, such as neutrophilia (Fig. 6A) and polyuria, were observed, no change in proviral load status occurred (Fig. 6B). In fact, some animals with detectable PBMC provirus showed a slight but insignificant decrease in proviral load after steroid treatment. Although steroid immunosuppression is relatively nonspecific, these results support the assertion that control of viral load in the three animals with constrained infection was not dependent upon normal immunologic function.

Tissue distribution of virus. Proviral tissue loads were examined at the end of the study in order to determine if (despite

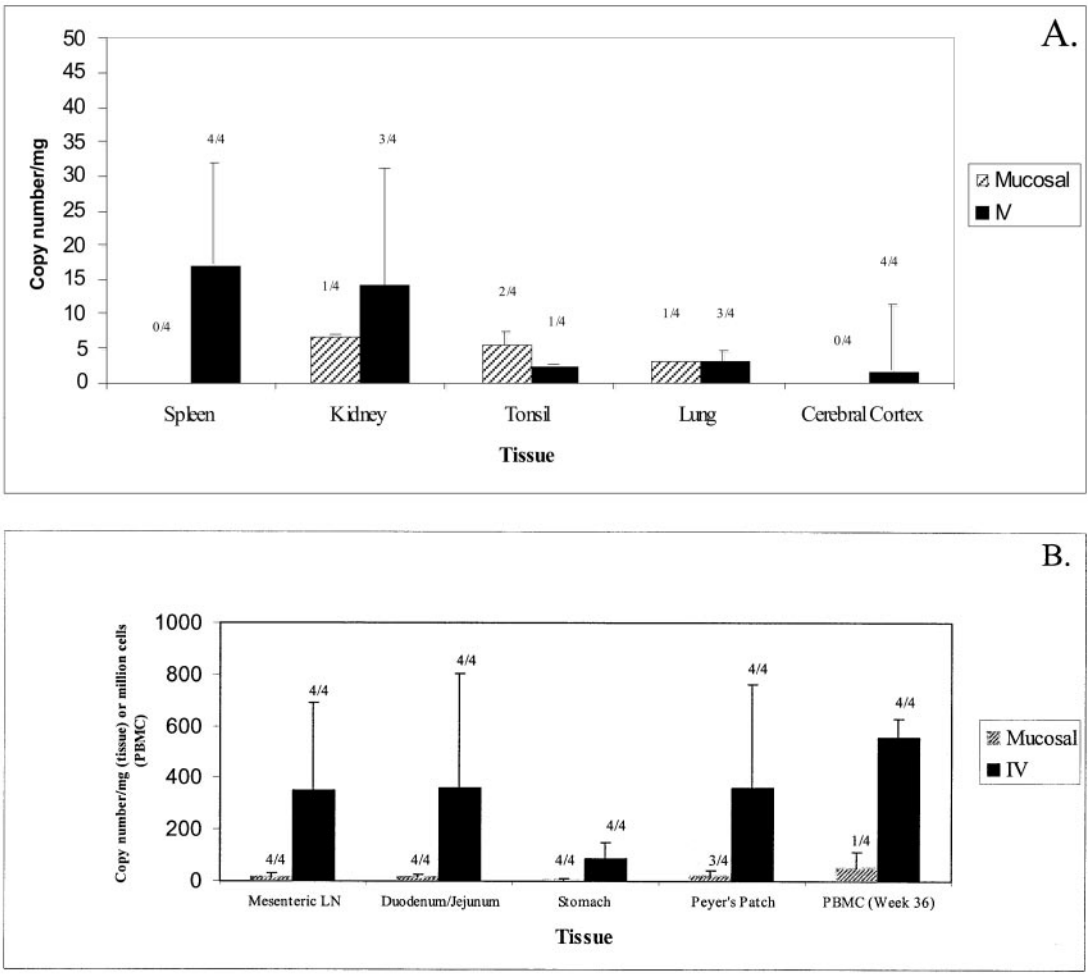

FIG. 7. PLV infection is enterotropic. Higher levels of provirus were found in the gastrointestinal tract and draining lymphatic tissue (B) than in lymphoid and other tissues (A) (note differences in scales). Bars represent the mean of all positive samples for each group reported. Lines above the bar represent 1 standard deviation. Numbers above the bars indicate the proportion of animals in the group in which virus was detected in the tissue sample. PBMC proviral burden at week 36 is also shown for comparison. No provirus was detected in control tissues obtained from PLV-negative cats.

undetectable virus in PBMCs) a tissue reservoir of PLV was present in the mucosally inoculated cats. Figure 7 shows the results of real-time PCR on tissues harvested at the termination of the study, 38 weeks after inoculation. PBMC proviral burden at week 37 is also shown for comparison. Surprisingly, provirus was not detected in mononuclear cells of bone marrow from any of the animals (data not shown), even in those animals retaining detectable proviral PBMC burdens. PLV was detected in spleen, kidney, lung, and brain of three or four of the i.v.-inoculated animals and only in the mucosally inoculated cat which demonstrated a similar PBMC proviral kinetics to the i.v.-inoculated group. Tonsil was provirus positive in only a few animals in each group. Mesenteric lymph node and jejunum were virus positive for all inoculated kittens, even those which had apparently cleared PBMC provirus. Stomach and Peyer's patches were positive in all i.v.-inoculated animals and in all but one of the mucosally inoculated animals tested, although the proviral load in PBMC proviral-negative animals was consistently lower (Fig. 7). The amount of provirus in gut-associated tissue was more than 10-fold higher than in spleen or tonsil in the i.v.-inoculated cats. These results indicated that PLV infection is not highly lymphotropic, and primary and residual infection may be localized to intestinal tract and draining lymph nodes.

DISCUSSION

As previously reported, domestic cats infected with PLV do not develop the clinical signs associated with FIV infection, which include CD4/CD8 lymphocyte inversion, chronic immune depletion, and opportunistic infections leading to death, similar to HIV infection of humans (48, 50). This study repeats these observations. Lymphadenopathy was observed at the onset of infection in all cats, and as previously noted, was transient in nature. The $CD4^+$ cell count decrease observed in the i.v.-inoculated group was also transient, and levels remained in the clinically normal range (>400 cells/μl) with the exception of one animal at two time points. Although this was a pilot study with small group sizes, we were able to determine that direct mucosal inoculation with this virus resulted in a high

rate of infection, although somewhat delayed in comparison to i.v. inoculation. Mucosal transmission is a likely mode in the natural host of PLV in the field and is the most common route for HIV infection. Since protective immunity at the mucosal surface is much different than that in the bloodstream, the fact that we are able to show consistent infection with mucosal inoculation of virus in the domestic cat model will enable extension of its use to vaccine trials as well as the study of natural transmission.

Mucosal infection of domestic cats was also nonpathogenic. The lack of clinical signs following i.v. or mucosal exposure to PLV could be attributed to in vitro passage of the viral inoculum; however, additional data suggest that this is not the case. We have recently observed that a MYA-1-passaged FIV stock was able to induce typical FIV disease in cats (J. A. TerWee and S. VandeWoude, unpublished data). Also, inoculation of a domestic cat with PBMC from a PLV-infected puma resulted in infection but no clinical signs, similar to observations we have made in this and other studies (39a, 51). Infections in domestic cats after experimental inoculation with FIV by oronasal, rectal, and vaginal routes have previously been described (12, 39); however, the virulence of infection is independent of the route of exposure. The cats used in this study were from a different source than other studies which have characterized virulent FIV, suggesting that genetic background may have played a role in our observations. However, recent studies in our laboratory using virulent FIV-C-PG as challenge virus have documented typical signs of FIV infection in cats from the same source used here (Cedar River Laboratory, Mason City, Iowa) (S. P. Troth, E. A. Hoover, J. A. TerWee, and S. VandeWoude, unpublished data). We controlled for genetic variability as much as possible in a study with small group sizes by separating siblings into different groups and randomizing genders among groups.

In the manuscript we describe an additional phenomenon in that kittens exposed via a mucosal route effectively eliminated detectable PBMC provirus. Although reduction of viremia and PBMC proviral load in the absence of antiviral therapy have been reported in cats infected with FIV (40; C. K. Mathiason, P. R. Avery, and E. A. Hoover, Abstr. 6th Int. Feline Retrovirus Res. Symp., abstr. PO-7, 2002), these reports have been relatively rare and are associated with very low proviral loads, lack of seroconversion, or neonatal transmission. Viremia after HIV, SIV, and FIV infection peaks early after infection and then declines to a steady-state level, presumably due to the initiation of a host immune response (19). Proviral load has been shown to follow a similar pattern in SIV and SHIV infections of monkeys (43) and in FIV infection in cats (42). PLV proviral loads were sustained throughout the 9-month study in four of four i.v.-inoculated and one of four mucosally exposed animals, but a gradual decline was noted over time, suggesting that these animals may have eventually eliminated PBMC provirus had they been monitored over a longer period of time.

Reduction in viremia or resistance to FIV, SIV, and HIV infections has been variously linked to parameters including a TH-1 cytokine profile, T-cell-mediated suppression, innate immunity, virus-specific CTL, or antibody responses (15, 26, 52). However, more recently a chronic robust immune response has been implicated in the immunopathology of immunodeficiency virus infection via activation-induced apoptosis of $CD4^+$ lymphocytes (18, 19, 41) and IFN-γ-induced inflammation (1). In this complex in vivo system, cause and consequence are difficult to assign. Clearance of infected PBMCs from mucosally inoculated cats was not associated with an earlier presence of antibody, cytokine response, pronounced proliferative response, or CTL activity. Steroid-induced immunosuppression did not result in an increase in circulating infected cells. In fact, it appeared that a few cats had a slight, though not significant, decrease in proviral load following steroid treatment. This has been previously reported and may be a result of immunosuppressive agent-induced death of the cells which the virus would normally target (38). Alternatively, this may signify that inappropriate immune stimulation is responsible for maintaining PBMC proviral loads, as has been hypothesized as a mechanism of primate lentivirus persistence (14). A significant antigen-specific proliferative response of lymphocytes was detected only at day 49 postinfection, a point by which proviral loads had already declined in mucosally exposed animals. It is possible that the PLV-infected cells in mucosally exposed animals were being cleared by an innate or mucosal immune response that we did not measure. It is also possible that our CTL assay was not sufficiently sensitive to detect CMI against a virus infecting only a small percentage of a subpopulation of PBMCs. Strong CTL responses in cats infected with FIV or other viruses, especially in assays without in vitro reticulation of cells, are not consistently documented in the literature.

Absence of a strong immune response to PLV infection correlating with absence of clinical signs seems to correspond to the response to infection of African monkeys with SIV. SIV-infected sootey mangabeys do not mount strong humoral or cellular antiviral immune responses against SIV. This lack of response had been hypothesized to restrict pathogenicity by reducing immune activation and apoptosis (14, 28). Infection of the natural host does not result in disease, and there is maintenance of normal $CD4^+$ cell counts despite high viral burdens (10, 14, 28). This supports the theory that most of the loss of $CD4^+$ cells in pathogenic HIV and SIV infections is due to bystander effects rather than direct killing of virus-infected cells, which represent a very small fraction of circulating $CD4^+$ lymphocytes (14, 18, 19, 41).

A greater level of apoptosis compared to controls has been documented in lymph nodes of FIV-infected animals (47), and altered cytokine expression (2, 3, 17, 33, 34) and an increased activation state of FIV-infected cells have been reported (46). However, one report of increased immune activation in FIV-infected cats by exposure to other viral and bacterial pathogens did not result in accelerated $CD4^+$ lymphocyte loss (44). Reduced responsiveness to mitogens and antigens has also been reported for PBMCs from FIV-infected cats (9, 35). We did not measure apoptosis in $CD4^+$ cells from PLV-infected cats, but we did not detect an alteration in cytokine balance in a limited number of cytokines representative of the TH1/TH2 response. While we did observe a sporadic decrease in mitogen response in infected animals compared to controls, we did not measure a deficit in proliferation to ovalbumin in infected animals, and ovalbumin-induced proliferation was strikingly higher than proliferation to PLV. These results indicate that $CD4^+$ cell function is maintained during PLV infection, once

again demonstrating a parallel between SIV-HIV and PLV-FIV.

Tissue distribution of virus in the mucosally inoculated group is also similar to SIV in apathogenically infected African monkeys. Up to 10^9 copies of RNA/million cells have been detected in the ileum and jejunum of African green monkeys (AGM) infected with SIV 4 years after infection, a point at which viral levels in secondary lymphoid tissues have declined (24). Apathogenically infected AGMs harbored significantly lower lymphoid tissue loads compared to pathogenically infected macaques, yet equivalent copy numbers per cell were noted in duodenum, jejunum, and colon (10). The gastrointestinal tract is also presumed to be a reservoir of HIV in humans (19). The data we present in this paper evaluate tissue provirus 9 months postinoculation, and at this point the highest levels of virus were found in the gut mucosa and lymph nodes. These tissues could represent a reservoir of replicating virus or could reflect the natural course of reduction of provirus, i.e., target tissues that are the site of initial high-level replication would be the last to clear virus. These findings contrast greatly with FIV infections of domestic cats, which result in high levels of provirus in bone marrow and other lymphoid tissues, with low levels of gut-associated provirus (6, 20; S. Troth and E. A. Hoover, Abstr. 7th Int. Feline Retrovirus Res. Symp., abstr. DT-1, 2004). PLV tissue distribution also differed from that of FIV in that we detected substantial disparity in proviral load between spleen and mesenteric lymph nodes and PLV tissue proviral load varied greatly between routes of inoculation (Troth and Hoover, Abstr. 7th Int. Feline Retrovirus Res. Symp., abstr. DT-1, 2004).

Intravenous inoculation of a high-titer PLV-containing supernatant resulted in earlier and higher proviral burdens than mucosal inoculation with a 1.25× volume of the same inoculum. The difference in proviral load kinetics between mucosally and i.v.-inoculated animals is likely influenced by the fact that a higher initial virus load is delivered to the PBMCs of i.v.-inoculated animals. Lower levels of viremia early after infection correlate with eventual viremic status and pathogenesis, consistent with the set point theory (29). This could be a result of an innate mucosal immune response, limited or different mucosal receptors for PLV, or selection of less "fit" virus across the mucosal barrier. However, it is unclear why, after initial establishment, proviral load was not amplified or sustained in mucosally infected animals.

This last point is a critical feature of this study which has no parallel in the FIV, HIV, or SIV literature. While extremely low levels of PLV provirus were measured by real-time PCR at the study end, no other detectable virus remained in the three animals which attained peak proviral loads of 10^4 to 10^5 copies/10^6 cells and developed antibody titers indicative of replicative virus. Our data indicate that this observation is not associated with any demonstrable host immune response that exceeded or differed from that in animals that remained persistently viremic, a finding with significant implications for HIV vaccine development and immune correlates of protection. Further studies will evaluate the role played by viral genotype selection and evolution in this important model of avirulent lentiviral clearance.

PLV infection of domestic cats is a versatile model of apathogenic lentiviral infection which will allow further investigation of lentiviral pathogenesis, including the role of immune activation and dysregulation in cell death, mechanisms of immune pathology and, perhaps most importantly, how viral clearance of lentiviruses may be achieved.

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