Human immunodeficiency virus type 1 can occasionally be detected as a cryptic or latent infection in seronegative, asymptomatic patients. To develop an animal model of host latency, cats were mucosally challenged with $10^7$–$10^{10}$ feline immunodeficiency virus (FIV)–infected T cells. Although high-dose exposure ($10^7$–$10^8$ T cells) resulted in progressive infection, no evidence of infection was seen in 5 of 6 cats exposed to lower numbers ($10^3$–$10^6$) of FIV-infected T cells. However, after ex vivo CD8$^+$ T cell depletion and phorbol myristate acetate treatment, FIV could be reactivated in tissues from 4 cats. Thus, latent tissue viral reservoirs can be induced by low-dose cell-associated mucosal challenge, providing a model to dissect the mechanisms that control reservoir establishment.

Cryptic HIV-1 infection, or host latency, is a poorly understood phenomenon that has primarily been described in cohorts engaged in high-risk sexual activity. In host latency, very low levels of HIV-1 DNA or inducible virus have been reported in seronegative, asymptomatic individuals years before seroconversion [1, 2]. This is similar to the occult infection reported in macaques after mucosal exposure to low doses of simian immunodeficiency virus (SIV) [3] and suggests that a state of latency may precede overt infection in some individuals.

The factors that control the establishment of host latency have been difficult to identify, because of the lack of a consistent animal model. We hypothesized that mucosal exposure to low numbers of infected cells would facilitate viral reservoir development and host latency. To address this, we used the well-described feline immunodeficiency virus (FIV) animal model of mucosal transmission, which mimics the pathogenesis of HIV-1 infection, including progressive, regressive, and transient infection [4]. We found that cats inoculated with intermediate to high numbers ($10^5$–$10^6$) of FIV-infected T cells developed progressive infection, whereas 5 of 6 of those exposed to lower numbers ($10^3$ or $10^4$) of FIV-infected T cells lacked traditional evidence of infection. Nevertheless, virus could be reactivated from tissues cultured from 4 of 5 seronegative cats. This suggests that latency and reservoir development are promoted after mucosal exposure to low numbers of infected T cells and provides a model to dissect the mechanisms of host latency.

**Methods.** Eighteen female cats were obtained from Liberty Labs and were cared for in accordance with the standards of the American Association of Accreditation of Laboratory Animal Care and the Institutional Animal Care and Use Committee. Cats were grouped (3 cats/group) and inoculated at 4 months of age with $10^5$ naive CD4$^+$ T cells or with serial dilutions of FIV NCSU,–infected CD4$^+$ T cells in 50 $\mu$L of lymphocyte medium. NCSU, is a clade A pathogenic FIV that does not produce high plasma viremia after mucosal transmission but induces high provirus levels and progressive immune dysregulation. Inoculum infectivity was demonstrated by immunocytochemistry (98% of cells positive), polymerase chain reaction (PCR) and coculture (positive at 1-cell dilution), and expression of Gag and Env proteins (data not shown). Blood was obtained before inoculation and at 2, 4, 8, and 12 weeks after inoculation. Spleen, small intestine, and popliteal, mesenteric, and medial iliac lymph nodes were harvested at 12 weeks after infection and were processed as described elsewhere [5].

Real-time PCR and reverse-transcription real-time PCR were performed in triplicate wells (ABgene), using the Mx3000 (Stratagene) to amplify a conserved region (170 bp) of FIV gag with the following primers: GagNCSU,–1247 sense (5′-GCTTAAAG-CAATTGAGCGAGGTAGTACTG-3′) and GagNCSU,–1417 antisense (5′-CCTCGAGATACCATGCTCTACACTGCATCC-3′). Standard curves were generated using serial dilutions of the plasmids pCR2.1-CCRS and pCR1-gag. Provirus level was expressed as number of gag copies per million CCR5 copies [5]. Plasma virus level was reported as copies per milliliter.

Concanavalin A (Con A)–induced lymphoproliferation was...
measured by tritiated thymidine uptake [6], quantified using a MicroBeta Jet scintillation counter (Wallac), and presented as the lymphoproliferation index: Con A (counts/min)/medium (counts/min). Serum antibody against FIV Gag p24 was detected by ELISA [6]. Titers were expressed as the inverse of the highest dilution that produced an OD of $\geq 0.1$ and $\geq 3$-fold the optical density of the cat’s prestudy sample. T cell subsets were analyzed by flow cytometry [6].

FCD4E indicator T cells were cultured with unfractionated peripheral blood mononuclear cells (PBMCs) and tissue lymphocytes or with PBMCs and tissue lymphocytes depleted of CD8+ T cells, for 6–10 days [5]. Twenty-four hours before cell harvesting, half the wells were treated with 0.2 μmol/L PMA. Total cell lysates were collected, and FIV gp120 was detected by Western blot analysis using SU1-30 (Custom Monoclonals International).

Analysis-of-variance models were used to assess differences among dose groups, with separate models for FIV provirus level and Con A–induced lymphoproliferation in PBMCs and each tissue type. Residual diagnostics were performed to evaluate the validity of each model, and $\log_{10}$ transformations of all response data were used to better satisfy assumptions of normality. Contrasts among groups in each model were assessed at a significance level of $\alpha = 0.05$. When multiple comparisons were performed within a model, the significance level was adjusted by the Bonferroni method. Statistical analyses were performed using SAS/STAT software (SAS System version 9.1 for Windows; SAS Institute).

Results. PBMC provirus and plasma viremia were detected as early as 2 weeks after infection in all cats challenged with $10^4$–$10^6$ FIV-infected T cells. Plasma viremia peaked at 8 weeks after infection, with no significant differences seen between groups (data not shown). At 12 weeks after infection, PBMC provirus level was $1 \log_{10}$ greater in the group inoculated with $10^4$ T cells (table 1) than in those inoculated with $10^5$ or $10^6$ T cells ($P < 0.001$) but did not differ significantly ($P = 0.51$) between the group inoculated with $10^5$ T cells and the group inoculated with $10^6$ T cells. The number of provirus copies in the gut was also greater in the group inoculated with $10^5$ T cells than in those inoculated with $10^6$ or $10^5$ T cells, although these differences were not statistically significant after Bonferroni adjustment (intraepithelial lymphocytes, $P = 0.057$; lamina propria lymphocytes, $P = 0.052$). Provirus was readily detected in spleen and lymph nodes, but the number of provirus copies was not significantly different between the dose groups ($P = 0.067–0.419$).

Consistent with FIV infection, cats in the $10^4$–$10^6$ T cell inoculation groups became seropositive and demonstrated a decrease in the CD4+:CD8+ T cell ratio (table 1). Con A–induced lymphoproliferation was significantly reduced in PBMCs and tissue lymphocytes, except for the iliac lymph node of cats from the $10^4$–$10^6$ T cell inoculation groups, and the greatest loss of proliferation occurred in the lamina propria (PBMCs, $P = 0.002$; spleen, popliteal lymph node, mesenteric lymph node, intraepithelial lymphocytes, and lamina propria lymphocytes, $P < 0.001$; iliac lymph node, $P = 0.138$).

In contrast, PBMC provirus, tissue provirus, and plasma viremia were not detected at any time in cats inoculated with $10^2$ or $10^3$ FIV-infected T cells, except for in cat ILC7 ($10^2$ T cell inoculation group; virologic data shown separately in table 1). In cat ILC7, very low numbers of provirus copies were detected only in the PBMCs and gut. Cats inoculated with $10^2$ or $10^3$ FIV-infected T cells remained seronegative and maintained their CD4+:CD8+ T cell ratio and Con A–induced lymphoproliferation ($P > 0.1$) (table 1), including ILC7, the cat in which low levels of provirus and plasma viremia had been detected. Although the optical density reading for cat ILC7 increased over time, it did not exceed the threshold for a positive signal.

To determine whether cats inoculated with $10^2$ or $10^3$ FIV-infected T cells harbored virus despite the lack of traditional markers, we first depleted CD8+ T cells from blood and tissue cell populations at 12 weeks after infection and then cocultured the total (nondepleted) and CD8+ T cell–depleted populations with uninfected CD4+ T cells. Expression of FIV gp120 was not detected in nondepleted PBMCs or tissues (figure 1A) but was detected in all tissues from all cats in the $10^2$ T cell inoculation group after CD8+ T cell depletion (figure 1B).

Phorbol ester up-regulates viral replication through activation of the protein kinase C pathway. Treatment with PMA induced expression of FIV gp120 in all tissues from all cats in the $10^2$ T cell inoculation group (figure 1C). CD8+ T cell depletion combined with PMA treatment appeared to synergistically increase gp120 expression (figure 1D). With the exception of ILC6 lamina propria, the intensity of the bands (as assessed by Kodak Digital Science 1D software) seen in the gut were 4- to 6-fold greater than that of the bands seen in PBMCs and spleen, whereas the bands in lymph nodes were of intermediate intensity (figure 1D).

After combination CD8+ T cell depletion and PMA treatment of samples from cats in the $10^2$ T cell inoculation group, gp120 was readily detected in PBMCs and tissues from cat ILC7 and in most tissues from cat ILE3 (figure 1E). However, we were unable to detect gp120 in any tissue from cat ILF4 (figure 1E).

Discussion. Latency is one strategy used by viruses to avoid clearance by the immune system. The term “latency” has more recently been used in the context of the HIV-1 system to describe the latent virus found within resting CD4+ T cells of HIV-1–infected individuals, but the concept of latency also includes situations in which exposed seronegative individuals harbor very low levels of virus. The presence of individuals who remain seronegative and asymptomatic despite HIV-1 exposure
Table 1. Summary of virologic and immunologic analysis at 12 weeks after infection.

<table>
<thead>
<tr>
<th>Inoculation group, cat</th>
<th>Provirus level, mean (SE), gag copies ×10^7/10^6 CCR5 copies</th>
<th>Mean CD4+:CD8+ T cell ratio</th>
<th>Mean IgG titer (×1000)</th>
<th>Con A–induced lymphoproliferation index, a mean (SE)</th>
<th>Detection of latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6 infected T cells</td>
<td>155^a (17)</td>
<td>32 (5)</td>
<td>25 (5)</td>
<td>35 (18)</td>
<td>ND</td>
</tr>
<tr>
<td>10^5 infected T cells</td>
<td>16^b (.9)</td>
<td>11^b (.3)</td>
<td>11^b (.3)</td>
<td>11^b (.8)</td>
<td>ND</td>
</tr>
<tr>
<td>10^4 infected T cells</td>
<td>14^c (4)</td>
<td>14^c (.8)</td>
<td>12^c (.3)</td>
<td>13^c (.8)</td>
<td>ND</td>
</tr>
<tr>
<td>10^3 infected T cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILE3</td>
<td></td>
<td>3.1</td>
<td>29 (5)</td>
<td>20 (2)</td>
<td>Latent</td>
</tr>
<tr>
<td>ILC7</td>
<td>.09</td>
<td>1.8</td>
<td>.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Con A, concanavalin A; IE, intraepithelial; LN, lymph node; LP, lamina propria; ND, not done; PBMCs, peripheral blood mononuclear cells.

a Con A (counts/min)/medium (counts/min).
b Significantly different from controls (P<.05).
c The 10^6 T cell inoculation group was significantly different (P<.05) from the 10^5 and 10^4 T cell inoculation groups for a given tissue.
d The 10^6 and 10^5 T cell inoculation groups were significantly different (P<.05) from the 10^4 T cell inoculation group but not from each other.
e Data on provirus level and detection of latency are given for individual cats ILE3, ILF4, and ILC7.
Feline immunodeficiency virus (FIV) latency and reactivation. FIV gp120 was detected in total lysates from cells of cats inoculated with $10^3$ (A–D) or $10^2$ (E) FIV-infected T cells after culture with CD4$^+$ T cells under several conditions: no treatment (A), CD8$^+$ T cell depletion (B), PMA treatment (C), and CD8$^+$ T cell depletion and PMA treatment (D and E). Lane 1, negative control; lane 2, positive control; lane 3, peripheral blood mononuclear cells (PBMCs); lane 4, spleen; lane 5, popliteal lymph node (LN); lane 6, mesenteric LN; lane 7, medial iliac LN; lane 8, intraepithelial lymphocytes; lane 9, lamina propria lymphocytes. The bottom panel shows β-actin as a loading control.

is well described in a recent review [7]. Similar observations of cryptic, occult, transient, regressive, and well-contained virus have been reported in SIV-exposed macaques [3, 8, 9] and FIV-exposed cats (reviewed in [4]). However, the immune, genetic, or exposure correlates that result in the ability to either resist infection or maintain virus at extremely low levels are unknown.

In our study, we found that the development of latency after mucosal exposure to infected cells appeared to be dose dependent. Low-dose vaginal exposure has also resulted in occult infection of some macaques [3] and has been hypothesized as a mechanism for the extreme containment seen in rare HIV-1–exposed seronegative individuals [10]. However, dose is unlikely to be the only critical factor, because others have shown that the infection seen after repeated low-dose SIV rectal exposure was similar to that seen for intermediate- and high-dose exposure [11].

Although virus has been transiently detected in other studies of cats [12] and rhesus macaques [8], we were unable to detect FIV DNA, plasma RNA, or seroconversion in latently infected cats. We did not screen for tissue provirus until 12 weeks after infection, so we may have missed early evidence of tissue infection; however, it seems more likely that viral latency was maintained throughout our study. Thus, our data seem to represent a further continuum of containment and are very similar to the findings of low levels of HIV-1 DNA in high-risk individuals years before their seroconversion [1]. We followed these cats for only 12 weeks, but we would predict, on the basis of studies of HIV-1 [1, 2] and SIV [3, 8], that they would remain seronegative with undetectable virus for a significant period before developing overt infection.

Our results strongly suggest that CD8$^+$ T cells play at least a partial role in the in vivo development of host latency. Several laboratories have recovered replication-competent HIV-1, FIV, and SIV from virus-negative samples after depletion of CD8$^+$ T cells (reviewed in [13]). CD8$^+$ T cell antiviral effects can be mediated through direct cytotoxic T lymphocyte lysis of virus-infected cells or the production of soluble factors that suppress viral replication or infection of new cells. We did not examine cytotoxic T lymphocyte activity, but previous studies [12] have demonstrated that FIV NCSU1 induces strong noncytolytic CD8$^+$ T cell activity, which is likely to be the antiviral effect we detected here.

Recent studies support the idea that cellular threshold and types of activation play a role in the establishment of FIV infection or latency in CD4$^+$ T cell populations [14]. In HIV-1–infected individuals, resting CD4$^+$ T cells are known to harbor latent HIV-1, but macrophages and other cells may also serve as reservoirs. In the present study, we used unsorted preparations of blood and tissue mononuclear cells for our in vitro depletion and culture assays; thus, latent virus may have been contained in either T cells or macrophages. Future studies will also be necessary to determine whether the latent populations differ in mucosal, systemic lymphoid, or circulating reservoirs.

CD4$^+$ T cells and Con A–induced lymphoproliferation were maintained in the blood and tissues of latently infected cats. This correlates with maintenance of CD4$^+$ T cells in the gut and mesenteric lymph nodes of vaccinated cats after FIV challenge, despite the ability to reactivate low levels of virus from these tissues [5]. The maintenance of Con A–induced lymphoproliferation was particularly significant in the lamina propria, a key target of lentiviral replication [15]. Interestingly, despite the detection of low numbers of provirus copies in the
gut of cat ILC7 (from the 10^2 T cell inoculation group). Proliferation in the lamina propria was not reduced, which suggests that there is a viral threshold necessary to induce immune dysfunction. Together, these findings suggest that near-complete suppression of viral replication may prevent bystander CD4^+ T cell death and maintain local immune function, even in the gut.

In summary, vaginal exposure to high numbers of FIV-infected cells resulted in classic systemic and mucosal infection. However, low-dose cell-associated exposure resulted in latent infection in 4 of 6 cats and in a well-contained infection in 1 additional cat. These data suggest that latent reservoirs can consistently be established after low-dose vaginal exposure to cell-associated FIV. Use of this model system will allow us to dissect the mechanisms of lentiviral latency and reservoir development.

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