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Infection of the choroid plexus by feline immunodeficiency virus

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The human, simian, and feline immunodeficiency viruses rapidly penetrate into the brain and trigger an inflammatory process that can lead to significant neurologic disease. However, the mechanisms that permit efficient trafficking of macrophage-tropic and the more neurotoxic lymphocytotropic isolates are still poorly understood. One potential source of virus entry may be the blood-CSF barrier provided by the choroid plexus. Infected cells are often detected within the choroid plexus but it is unclear whether this reflects trafficking cells or infection of the large macrophage population within the choroidal stroma. To address this issue, we cultured fetal feline choroid plexus and evaluated the ability of feline immunodeficiency virus (FIV) to establish a primary infection. Significant provirus was detected in macrophage-enriched choroid plexus cultures as well as in the choroid plexus of cats infected *in vivo*. FIV p24 antigen production *in vitro* was very low but detectable. Addition of a feline T-cell line to macrophages inoculated with FIV resulted in a dense clustering of the T cells over macrophages with dendritic cell-like morphologies and a robust productive infection. The direct infection of choroid plexus macrophages with FIV, the efficient transfer of the infection to T cells indicate that the choroid plexus can be a highly efficient site of viral infection and perhaps trafficking of both macrophage-tropic and T-cell-tropic viruses into the CNS. *Journal of NeuroVirology* (2002) **8,** 211–224.

Keywords: human immunodeficiency virus; macrophages; brain; AIDS; neural culture

Introduction

It is now well established that the human immunodeficiency virus (HIV-1), the lentivirus associated with the acquired immunodeficiency syndrome (AIDS), is both neuroinvasive and neurovirulent, producing in many patients a central nervous system (CNS) disease that has become a common cause of dementia in individuals under the age of 60 (Janssen, 1991). Although infection of the parenchyma is considered a critical event in AIDS-related neuropathogenesis, it is still unclear how brain viral burden initially evolves and how it is ultimately sustained. There is now considerable evidence that microglia and macrophages represent the primary viral reservoirs within the CNS (Wiley *et al*, 1986; Takahashi *et al*, 1996) and that these cells are predominantly infected by lentiviral isolates that require beta chemokine receptors, particularly CCR5 and CCR3, as entry cofactors (He *et al*, 1997; Shieh *et al*, 1998; Albright *et al*, 1999). However, recent studies, comparing the

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ability of different HIV-1 isolates to induce apoptosis in neural cultures (Ohagen *et al*, 1999; Zheng *et al*, 1999), have revealed that significant neurotoxicity was produced not by brain-derived isolates but rather blood-derived strains, ones that typically infect lymphocytes via CD4 and the alpha chemokine receptor, CXCR4. Although previous investigations have characterized systemic and local sources that may contribute to CNS viral burden, none of these studies have offered direct documentation of significant lymphocyte infiltration in the brains of HIV-1-infected patients. Thus the mechanisms by which these particularly neurotoxic lymphocytotropic isolates might invade the CNS remain currently unclear.

One potential route for trafficking of lymphocytotropic isolates may be the choroid plexus, a highly vascularized structure distributed throughout the cerebral ventricles that provides a barrier between the peripheral circulation and the cerebrospinal fluid (CSF). Multiple studies have documented the presence of viral nucleic acid or antigen within the choroid plexus stroma of HIV-1-infected patients (Falangola *et al*, 1995; Hanly and Petito, 1998; Petito *et al*, 1999), SIV-infected nonhuman primates (Lackner *et al*, 1991; Lane *et al*, 1996), and FIV-infected cats (Beebe *et al*, 1994). Hanly and Petito (1998) have further demonstrated that these virus-positive cells may display a prominent dendriform morphology and intense immunoreactivity for class II MHC and HLA-DR, consistent with other reports of putative dendritic cells (DCs) within the choroid plexus (Matyszak and Perry, 1996; Serot *et al*, 1997; McMenamin, 1999; Serot *et al*, 2000). There is an increasing recognition that DCs may make a significant contribution to systemic infection by HIV-1 (Clark, 1996; Knight and Patterson, 1997; Pope, 1999), and the possibility that they may also be found within the choroid plexus could have similarly important implications for dissemination of virus in the CNS. Indeed, the choroid plexus could potentially represent a major site at which lymphotropic virions are produced, given 1) the high efficiency with which DCs may transfer infection to CD4+ T-lymphocytes (Cameron *et al*, 1992; Granelli-Piperno *et al*, 1998); and 2) the frequent observations of lymphocytic infiltrates within the choroid plexus of lentivirus-infected hosts (Dean *et al*, 1993; Beebe *et al*, 1994; Falangola *et al*, 1995; Czub *et al*, 1996a, 1996b; Hanly and Petito, 1998). Moreover, if infiltrating T-lymphocytes are in fact seeded with virus by infected choroid plexus cells, they could potentially release progeny virions into the CSF and thereby distribute these particularly neurotoxic viral isolates throughout all levels of the neuraxis.

However, this model of neuroinvasion remains largely speculative, as several fundamental questions about choroid plexus infection remain currently unanswered. In particular, the previous descriptions of lentiviral transcripts and protein within the choroid plexus do not clearly establish the presence of a productive infection. Given the emerging evidence that dendritic cells may possess novel mechanisms for capturing and retaining virions (Geijtenbeek *et al*, 2000), it is possible that virus may be passively "trapped" on the surface of these putative DC-like cells. Thus any evaluation of the choroid plexus' potential to serve as an actual lentiviral reservoir will first require a better understanding of its capacity to support either viral replication or transfer of virus to T-lymphocytes. For this purpose we have established primary cultures of feline choroid plexus and examined the response to three neurovirulent isolates of the feline immunodeficiency virus (FIV), a lentivirus frequently used as a model for AIDS-related neurologic disease. FIV is structurally similar to both HIV-1 and SIV, shares similar cellular targets (Brunner *et al*, 1989; Dow *et al*, 1990; English *et al*, 1993; Beebe *et al*, 1994; Dean *et al*, 1996; Dow *et al*, 1999), and like the primate lentiviruses produces a clinical syndrome that frequently includes severe neurologic deficits (Phillips *et al*, 1994, 1996; Podell *et al*, 1993, 1997). The results from the present study offer additional confirmation that the choroid plexus may be infected by lentiviruses and further illustrate how this structure could potentially serve as an important viral reservoir and site for viral and immune cell trafficking into the CNS.

Results

Primary cultures of feline choroid plexus

Representative examples of feline choroid plexus cultures are shown in Figure 1. Small explants of weakly adherent choroid plexus were present which contained tufts of healthy epithelium (Figure 1A). At higher magnification, cuboidal epithelial cells could be visualized surrounding the choroid plexus stroma (Figure 1B). Choroid plexus macrophages were identified within intact explants adjacent to the epithelium (Figure 1C) and throughout the stroma (not shown) by uptake of acetylated low density lipoprotein conjugated to the fluorescent dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI-acyl-LDL; Biomedical Technologies; 2.5 μg/ml in DMEM). Macrophages were often seen extending processes through the epithelial layer (epiplexus cells, Figure 1D). Macrophages rapidly migrated from the explants and, after 8 days *in vitro*, choroid plexus cultures were enriched in macrophages with multiple phenotypes, including large, flattened cells as well as smaller, activated cells containing vacuoles and cytoplasmic processes (Figure 1E). Under these conditions, the explant was no longer adherent and few epithelial cells remained by 8 days.

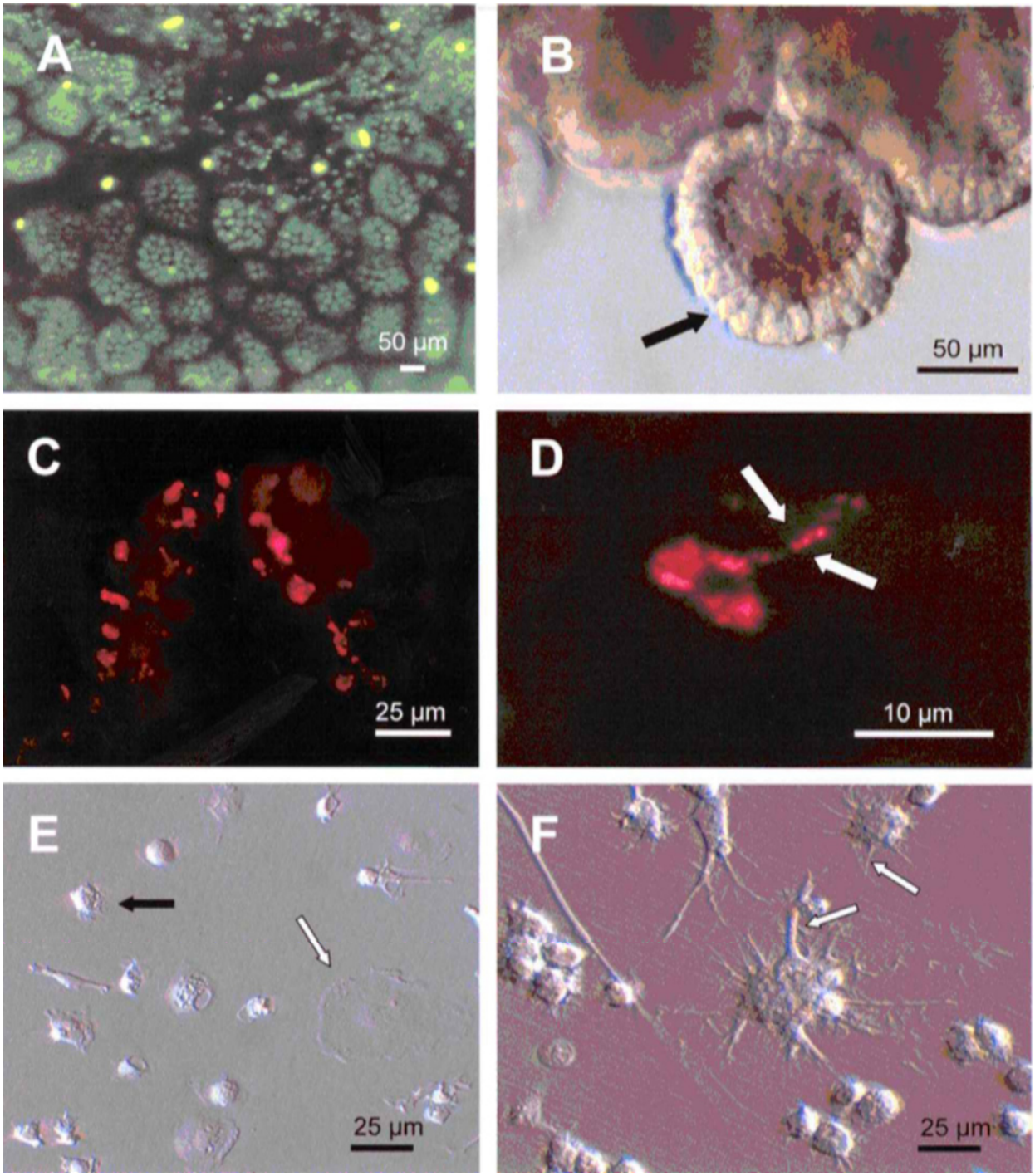

Figure 1 Primary cultures of uninfected feline choroid plexus. (**A**) Choroid plexus explant stained after 30 days *in vitro* with Syto-16, a marker that labels the nuclei of live cells. At this time, the explant survives in culture as a suspended group of cells. (**B**) Cuboidal epithelial cells (black arrow) overlying choroid plexus stroma in an intact tissue explant dissected from fetal cat brain. (**C**) Choroid plexus macrophages in an intact explant labeled by uptake of DiI-acyl-LDL. Many labeled macrophages in the explant are closely associated with the cuboidal epithelium (unstained). (**D**) Close-up of an epiplexus cell in a choroid plexus explant, labeled with DiI-acyl-LDL, extending a single process between (unstained) adjacent epithelial cells (white arrows). (**E**) During the initial development of these cultures, the macrophages appear to migrate from the choroid plexus explant and attach to the bottom surface of the plate where multiple phenotypes can be seen. Some cells display a rounded morphology with small cytoplasmic processes (black arrow) and robust phagocytic activity, while other cells appear more flattened (white arrow). (**F**) Choroid plexus macrophages exhibiting a characteristic , dendriform morphology following a 24-h stimulation with rhuCD40-ligand trimer (rhuCD40-L). By 48 h postchallenge with rhuCD40-L, cells frequently extend numerous, pronounced processes from the perikaryon (white arrows).

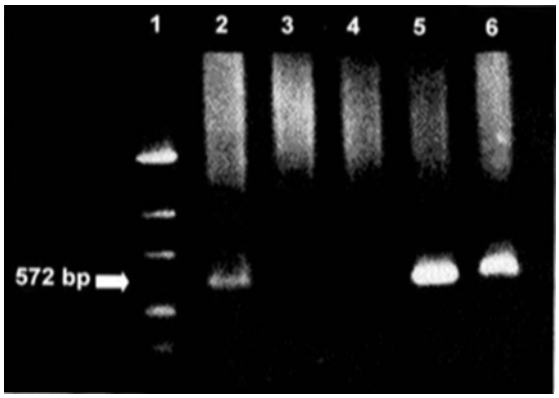

Figure 2 Nested PCR amplification of FIV proviral DNA sequences using primers against the viral *gag* gene. Lane 1, DNA mass ladder; lanes 2–6: provirus amplified from DNA extracted from: Crandell feline kidney (CrFK) cells, chronically infected with FIV, used as a positive control (lane 2); uninfected feline CD4+ T-lymphocytes, used as a negative control (lane 3); choroid plexus inoculated *in vitro* with boiled FIV-$NCSU_1$ (lane 4); choroid plexus inoculated *in vitro* with FIV-$NCSU_1$ (lane 5); and choroid plexus dissected from a cat at necropsy that developed feline AIDS following experimental infection with FIV-$NCSU_1$ (lane 6).

Estimates of the relative purity of the macrophage cultures at his point indicated that a minimum of $91 \pm 11\%$ of the cells were macrophages based on morphology and staining with DiI-Ac-LDL. Contaminating cells consisted primarily of epithelial cells and fibroblasts. After 24-h stimulation with CD40L, a subset of choroid plexus macrophages often displayed a prominent dendriform morphology with numerous, well-defined processes extending from the perikaryon (Figure 1F). Robust proliferation of the macrophages in these treated cultures resulted in an even greater proportion of macrophages to non-macrophages resulting in enriched cultures.

Detection of FIV provirus in choroid plexus cultures by nested PCR

FIV proviral DNA sequences were detected in extracts prepared from choroid plexus cultures inoculated with FIV-$NCSU_1$ (Figure 2). In addition, provirus was successfully amplified from DNA extracted from choroid plexus dissected postmortem from a cat that developed feline AIDS after experimental infection with FIV-$NCSU_1$. Detection of provirus required nested PCR amplification using both external and internal primers against a conserved region of the FIV gag gene. Amplification with external primers alone was not sufficient to produce a product that could be identified by gel electrophoresis (data not shown). Control cultures inoculated with heat-inactivated FIV were consistently negative for provirus.

FIV replication in choroid plexus cultures

Viral replication in choroid plexus cultures was determined by the presence of the p24 core antigen in the culture supernatants (Figure 3). Choroid plexus cultures infected with FIV showed a very slight

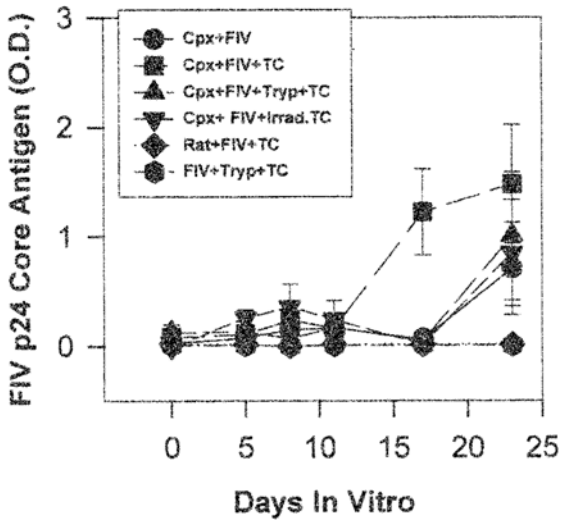

Figure 3 Viral replication in choroid plexus cultures inoculated with FIV. The FIV p24 core antigen was measured in cell-free tissue culture supernatants by ELISA. Results were quantified by spectrophotometry and expressed as optical densities (O.D.). Temporal profiles are shown for the following conditions: choroid plexus cultures inoculated with FIV (Cpx+FIV); choroid plexus inoculated with FIV and cocultured with CD4+ T-lymphocytes on day 11 (Cpx+FIV+TC); choroid plexus treated with trypsin following inoculation with FIV, then cocultured with CD4+ T-lymphocytes on day 11 (Cpx+FIV+Tryp+TC); choroid plexus inoculated with FIV and cocultured with irradiated CD4+ T-lymphocytes on day 11 (Cpx+FIV+Irrad.TC); rat neural cultures (nonpermissive control) inoculated with FIV and cocultured with CD4+ T-lymphocytes on day 11 (Rat+FIV+ TC); and CD4+ T-lymphocytes inoculated with an FIV stock predigested with trypsin (FIV+Tryp+TC). Temporal profiles represent averages of three individual observations.

increase in p24 production at day 8 postinoculation that, in the absence of the indicator cell line, decreased to negligible levels at days 11 and 17. By day 23 postinoculation, p24 antigen levels were significantly increased above baseline. Addition of FCD4-E cells to choroid plexus cultures at day 11 resulted in an increased production of p24 at days 17 and 23. This increase was also observed at day 23 in cocultures of FCD4-E cells and choroid plexus that had been treated with trypsin after inoculation with FIV. No p24 production was detected in FCD4-E cultures inoculated with a trypsinized viral stock, confirming that enzyme treatment was sufficient to digest cell-free virus. In addition, viral antigen was never detected in rat cortical neural cultures exposed to FIV, either before or after addition of the indicator cells.

The three viral isolates used in this study were all capable of replication within choroid plexus cultures (Figure 4). Choroid plexus inoculated with either FIV-MD or FIV-PPR showed similar increases in p24 production 12 days after coculture with FCD4-E cells. A slightly different kinetic profile was observed in cocultures inoculated with FIV-$NCSU_1$, which achieved maximal levels of viral core antigen in the supernatant by day 17, with a slight decrease observed at day 23.

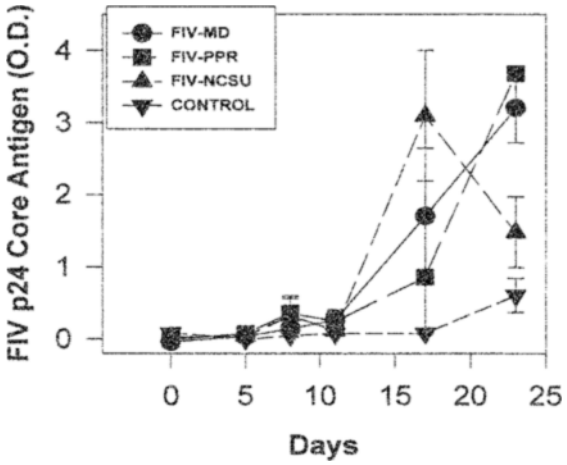

Figure 4 Viral replication in choroid plexus cultures inoculated with either FIV-MD, FIV-PPR, or FIV-$NCSU_1$ and cocultured with CD4+ T-lymphocytes on day 11. The FIV p24 core antigen was measured in cell-free tissue culture supernatants by ELISA. Results were quantified by spectrophotometry and expressed as optical densities (O.D.).

Examination of the cocultures by light microscopy revealed the presence of large cell clusters, which typically formed within 24 h after seeding choroid plexus cultures with FCD4-E cells (Figure 5A). By 48 h, syncytia were frequently observed (Figure 5B). Although choroid plexus macrophages readily attached to the coverslip, FCD4-E cell clusters occasionally detached from the substrate and were observed floating in the culture medium. Because of this, the large bound aggregates were often depleted from the infected cocultures by the end of the experiment.

To identify the cell type underlying the choroid plexus-FCD4E cell aggregates, choroid plexus macrophages were pre-loaded with the fluorescent marker, DiI-acyl-LDL, immediately prior to addition of the indicator cells. Highly fluorescent macrophages were consistently observed deep within these clusters (Figure 5C). Clustering of the FCD4E cells was highly specific to a subpopulation of macrophages. Many macrophages failed to support clustering of the FCD4E cells (e.g., Figure 5C upper left) and no evidence was detected that FCD4-E cells bound to fibroblasts or the small amount of choroidal epithelium present *in vitro*.

Identification of FIV-infected choroid plexus cells by immunohistochemistry

Although every effort was made to maintain FCD4-E cells in cocultures for immunohistochemical analysis, the majority of these nonadherent cells did not remain once coverslips were fixed in paraformaldehyde and washed in buffer. Nevertheless, a small number of cells were successfully retained, allowing a comparison of the relative levels of p24 antigen within the different cell types present *in vitro*. In cocultures of FCD4-E cells and choroid plexus macrophages inoculated with FIV, robust staining was observed in the FCD4-E cells, which could be clearly distinguished from the macrophages, fibroblasts, or epithelial cells by their small size (Figure 6A). Small clusters of heavily stained FCD4-E cells were seen adjacent to unstained or weakly stained macrophages

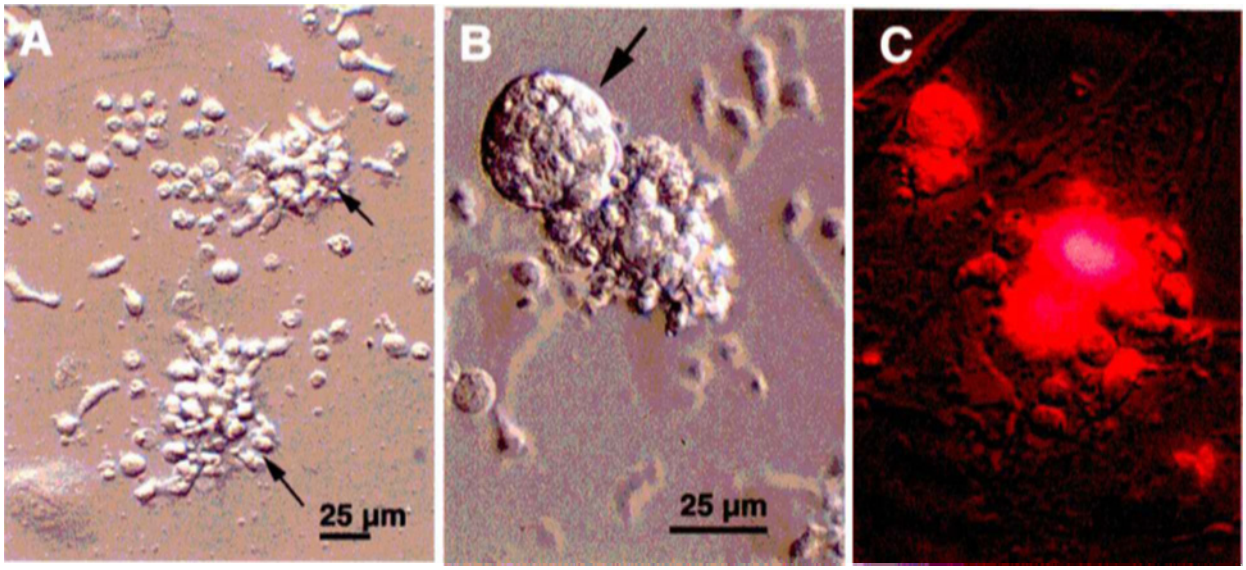

Figure 5 Cocultures of choroid plexus inoculated with FIV followed by addition of the feline CD4+ T-lymphocyte cell line, FCD4-E. (**A**) Brightfield micrograph depicting large cell clusters (arrows) that form within 24 h following addition of FCD4-E cells to choroid plexus cultures. Dendritic-like processes can be seen extending from underneath the cell cluster. (**B**) By 48 h, large syncytia (arrow) are observed in cocultures of FCD4-E cells and choroid plexus pre-inoculated with FIV. To determine the choroid plexus cell type underlying these cell clusters, FCD4-E cells were added to choroid plexus cultures preloaded with DiI-acyl-LDL to label choroid plexus macrophages. A representative cluster is shown under combined brightfield/fluorescence optics (**C**), demonstrating that the cell underlying the cluster has been labeled with the fluorescent marker.

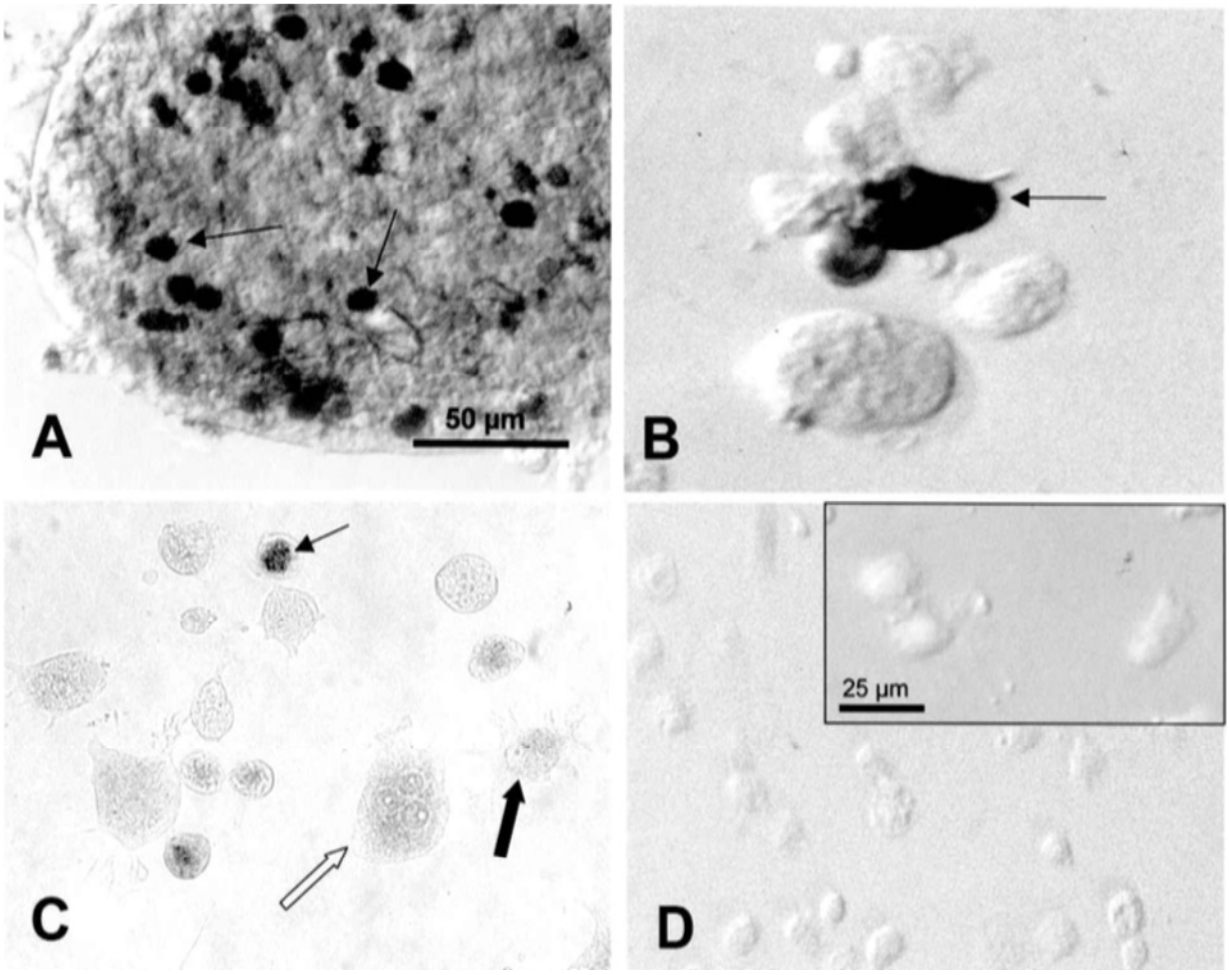

Figure 6 Identification of FIV-infected cells by immunohistochemistry for the p24 core antigen. (**A**) Robust staining was observed in FCD4-E cells (white arrows) cocultured with choroid plexus that had been inoculated with FIV. FCD4-E cells can be distinguished from the multiple choroid plexus cell-types by their small size. In this example, heavily labeled FCD4-E cells are shown attached to a large cluster of cells that are also immunoreactive. (**B**) A cell cluster intensely stained for the p24 core antigen (white arrow). Other adjacent cells in the culture show little or no immunoreactivity. (**C**) p24 immunoreactivity in choroid plexus macrophages inoculated with FIV but not cocultured with FCD4-E cells, viewed under oil immersion optics. Foci of immunoreactivity (small arrow) are seen within a subset of macrophages as well as within a dendritic-like choroid plexus cell (black arrow) and a multinucleated macrophage-like cell (white arrow) (**D**) Uninoculated choroid plexus macrophages show almost no labeling by the anti-p24 mAB. Infected choroid plexus macrophages incubated with preimmune serum in place of primary antibody also showed no staining (inset). Contrast is enhanced in the controls to allow visualization of the cells. Magnification is the same for all figures.

(Figure 6B). Because of the density and intensity of the stained FCD4-E cells, it was not possible to determine if underlying macrophages were FIV-positive. Isolated macrophages within these cocultures also exhibited staining (Figure 6C) that was significantly above background levels observed in uninfected cultures (Figure 6D) or cultures incubated with pre-immune serum in place of primary antibody (Figure 6D, inset). The staining was visible as foci of intracellular reaction product as well as an increased level of diffuse cytoplasmic reaction product. To compare the levels of macrophage staining intensity in different culture conditions, densitometric analysis was performed on cells in choroid plexus cultures inoculated with FIV; choroid plexus cultures inoculated with FIV and seeded with FCD4-E cells; and uninoculated choroid plexus cultures seeded with FCD4-E cells. Figure 7 depicts the frequency distribution of density values for macrophages in each of these three conditions. A chi-square analysis indicated that a significant increase in staining intensity was observed in FIV(+) choroid plexus macrophages when compared to cultures not exposed to virus ($\chi^2 = 42$; $P < 0.0001$; df = 6). A significant increase in mean optical density was also detected in FIV+ choroid plexus macrophages cocultured with FCD4-E cells compared to FIV(+) macrophages cultured alone ($\chi^2 = 24.8$; $P = 0.0004$; df = 6).

Discussion

In the present study we examined the capacity of the choroid plexus to support viral entry and

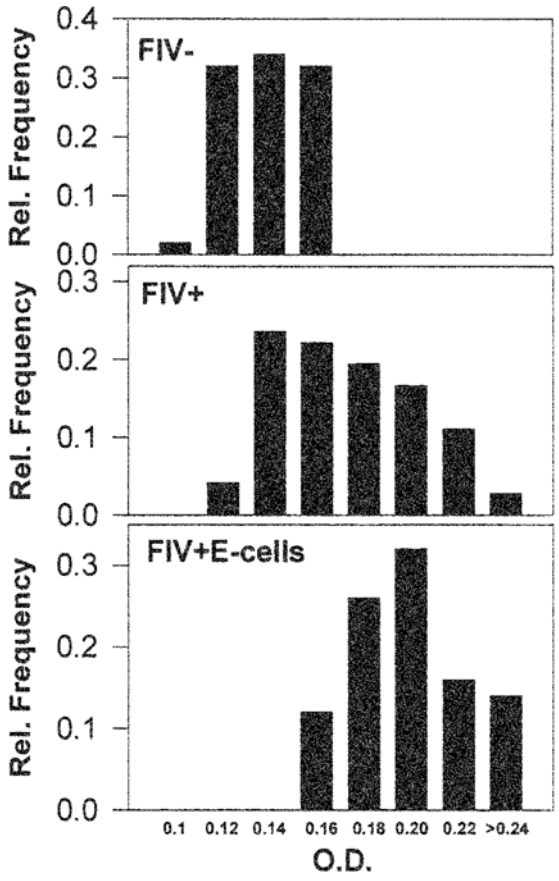

Figure 7 Frequency analysis of p24 immunoreactivity in uninoculated choroid plexus macrophages (FIV−), FIV-inoculated choroid plexus macrophages (FIV+), and FIV-inoculated choroid plexus macrophages cocultured with FCD4-E cells (FIV+TC). Digital images were captured for cells in each condition, and average optical densities were calculated for each cell based on the average gray scale value. The frequency distribution of cellular OD values was analyzed by a chi-square test. A significant difference was observed between the FIV+ and FIV− populations ($\chi^2 = 42.0$; $P < 0.0001$; df = 6), and between the FIV+ choroid plexus cultures in the presence (FIV+TC) and absence (FIV+) of FCD4-E cells ($\chi^2 = 24.8$; $P = 0.0004$; df = 6).

replication of three isolates of FIV whose neurovirulence has been well documented. Cats experimentally infected with FIV-MD exhibit increased cortical slow wave activity in quantitative electroencephalograms (Phillips *et al*, 1994, 1997, Podell *et al*, 1993, 1997, 1999), prolonged latencies in brainstem-evoked potentials (Phillips *et al*, 1994; Podell *et al*, 1997), alterations in sleep architecture (Prospero-Garcia *et al*, 1994), and performance deficits in multiple behavioral tasks (Steigerwald *et al*, 1999). FIV-PPR produces a comparable pattern of neurologic sequelae *in vivo* (Phillips *et al*, 1996; Barr *et al*, 2000) and may also induce neurotoxicity *in vitro* by disrupting neuronal calcium homeostasis (Gruol *et al*, 1998) and decreasing glutamate uptake by astrocytes (Billaud *et al*, 2000). In addition, previous work in our laboratory has shown that inoculation with FIV-$NCSU_1$ results in significant neuronal loss *in vivo* (Meeker *et al*, 1997) as well as enhanced excitotoxicity (Meeker *et al*, 1996) and microglial proliferation (Meeker *et al*, 1999) *in vitro*. Taken together these observations demonstrate that, like HIV-1, FIV infection may be associated with a neurologic disease which in many respects can be recapitulated in various neural cell culture systems.

The primary cultures described in this report provide an *in vitro* system in which critical interactions between lentiviruses and the choroid plexus may be further characterized. Feline choroid plexus cultures consisted principally of a heterogeneous population of macrophages, some of which displayed a stellate morphology characteristic of the putative dendritic cells (DCs) proposed to exist in choroid plexus (Matyszak and Perry, 1996; Serot *et al*, 1998; McMenamin, 1999). The multiple, elongated processes of these cells became more pronounced following stimulation with CD40-ligand, a member of the tumor necrosis factor (TNF) superfamily (Armitage *et al*, 1992) previously shown to promote survival and activation of DCs (Caux *et al*, 1994). Moreover, the aggregation we observed between choroid plexus cells and a feline CD4+ T-lymphocyte cell line was similar to the large conjugates that form between systemic DCs and T-lymphocytes (Flechner *et al*, 1988; Pope *et al*, 1995a). Although the process-bearing choroid plexus macrophages may share morphological and functional properties with DCs, we were unable to characterize their immunophenotype due to the lack of reagents that recognize the feline homologues of DC-specific surface antigens. Thus in our evaluation of viral replication kinetics, we have collectively referred to this cell population as macrophages, while acknowledging that a subset display features suggestive of DCs.

Virus entry and reverse transcription in choroid plexus cells was demonstrated by the presence of proviral DNA sequences. Provirus in choroid plexus cultures inoculated with FIV-$NCSU_1$ was identified via nested polymerase chain reaction (PCR) using *gag*-specific primers. These results support recent findings of Petito *et al* (1999), who used nested PCR to amplify *env* sequences in choroid plexus removed at autopsy from patients infected with HIV-1. The requirement for nested PCR amplification may indicate that proviral sequences are present only in low copy numbers. Similar results have been reported by Pope *et al* (1995b), who demonstrated that purified DCs pulsed *in vitro* with HIV-1 may carry less than 100 copies of proviral DNA per 5×10^4 cells, the approximate number of choroid plexus macrophages derived from a single explant *in vitro*. Yet this study provided convincing evidence that, even with this low level of infection, virus-pulsed DCs were capable of transmitting a vigorous, cytopathic infection to CD4+ T-lymphocytes. Thus, the possibility that the choroid plexus might harbor a relatively low proviral burden does not diminish the potential significance of the infection.

Virus release in choroid plexus cultures was monitored by assaying culture supernatants for the FIV p24 core antigen. Increased levels of p24 were detected following addition of the CD4+ T-lymphocyte cell line, FCD4-E, to choroid plexus cultures inoculated with all three FIV isolates. It is unlikely that this viral replication could be attributed to infection of the T-lymphocytes by residual inoculum, because no viral antigen was detected in nonpermissive rat neurons inoculated with FIV and cocultured with FCD4-E cells; and digestion of remaining cell-free virions with trypsin prior to addition of FCD4-E cells failed to abolish the increased p24 production. Small increases in p24 levels were observed at days 8 and 23 in FIV+ choroid plexus cultures not seeded with indicator cells, suggesting that the choroid plexus might support a low grade productive infection even in the absence of T-lymphocytes. Similar kinetics have been seen for monocyte-derived macrophages infected with FIV but with higher levels of virus production and no apparent biphasic pattern (Dean *et al*, 1999). In contrast, the high levels of virus production within 8–15 days after addition of FCD4E-cells suggests that transfer of infection may be a more significant contribution of the choroid plexus rather than macrophage virus production alone. The increased viral antigen levels detected in cocultures most likely reflect protein synthesis primarily in FCD4-E cells, because these increases were observed in cocultures of choroid plexus with intact, but not irradiated, FCD4-E cells.

Identification of FIV-infected cells by immunohistochemistry confirmed that production of viral antigen in cocultures occurred predominantly in the CD4+ T-lymphocytes, which were clearly distinguished from the multiple choroid plexus cell types by size. Robust staining was also observed in large cell clusters, although it could not be determined whether these labeled aggregates contained both FCD4-E cells and choroid plexus macrophages or if they represented syncytia formation by infected FCD4-E cells alone. In addition, densitometric analysis of the immunohistochemistry results revealed significant increases in staining intensity in isolated FIV(+) choroid plexus macrophages versus uninoculated control cultures, and isolated FIV(+) choroid plexus macrophages in cocultures compared to macrophages exposed to virus alone. These results lend additional support to the conclusion that a low-grade productive infection was achieved in feline choroid plexus macrophages inoculated with FIV. The data further indicates that addition of feline CD4+ T-lymphocytes may stimulate viral replication in infected choroid plexus macrophages, although the relative level of viral antigen in macrophages remained substantially lower than the antigen levels detected in either T-lymphocytes or large cell aggregates. Most efforts to identify infected cells within the choroid plexus have localized viral protein or transcripts in mononuclear cells within the choroid plexus stroma (Lackner *et al*, 1991; Falangola *et al*, 1995; Hanly and Petito, 1998; Petito *et al*, 1999). However, Harouse *et al* (1989) and Bagasra *et al* (1996) have suggested the presence of a latent HIV-1 infection in choroid plexus fibroblasts and epithelial cells, respectively. In the present study, FIV+ choroid plexus cells labeled *in vitro* with an anti-p24 mAB all resembled the infected stromal macrophages and perhaps DCs that have been described in previous studies of HIV-1-infected patients (Falangola *et al*, 1995; Hanly and Petito, 1998; Petito *et al*, 1999). Although our data do not exclude the possibility that the few choroid plexus fibroblasts or epithelial cells in these cultures may harbor a latent infection, we saw no evidence of CD4-E cell binding to or infection of these cells.

The demonstration by Hanly and Petito (1998) that virus-positive cells within the choroid plexus exhibit ultrastructural and immunophenotypic features characteristic of DCs raises important questions about the nature of choroid plexus infection. The DC-system of antigen presenting cells includes distinct subpopulations (Banchereau and Steinman, 1998), which may display different susceptibilities to, and capacities to support, productive infection (Cameron *et al*, 1994, 1996; Schuurman *et al*, 1995; Grouard and Clark, 1997; Knight and Patterson, 1997). Thus viral dynamics within the choroid plexus could potentially be shaped by the ability of these DC-like cells to function as either the follicular DCs within germinal centers or the DC populations isolated from blood or skin. Follicular DCs do not typically support a productive infection (Schmitz *et al*, 1994; Tenner-Racz *et al*, 1994) yet may harbor a significant, persistent viral burden by retaining infectious virions on their cell surface (Pantaleo *et al*, 1993; Schmitz *et al*, 1994; Haase *et al*, 1996). In contrast, blood- and skin-derived DCs may be productively infected by divergent HIV-1 isolates (Ayehunie *et al*, 1997; Blauvelt *et al*, 1997; Sozzani *et al*, 1997; Granelli-Piperno *et al*, 1998; Rubbert *et al*, 1998) and may form conjugates with CD4+ T-lymphocytes in which substantial viral replication occurs (Cameron *et al*, 1992; Pope *et al*, 1995a, 1995b). This particular pattern of infection most closely resembles the profile we observed in feline choroid plexus cultures inoculated with FIV. Nevertheless, the slowing of the kinetics of viral replication by treatment with trypsin also suggests that virions bound to the cell surface may contribute to the transfer of infection to the FCD4-E cells.

Our observations of viral replication in choroid plexus/lymphocyte cocultures raise the possibility that choroid plexus infection *in vivo* could provide an efficient mechanism for infection of trafficking T-lymphocytes. This possibility is highlighted by reports of lymphocytic infiltrates within the choroid plexus stroma of HIV-1-infected patients (Falangola *et al*, 1995; Hanly and Petito, 1998), SIV-infected nonhuman primates (Dean *et al*, 1993; Czub *et al*, 1996a, 1996b), and FIV-infected cats (Beebe *et al*,

1994). Under these conditions, one might predict that virus recovered from the choroid plexus of an infected host would be a heterogeneous pool that includes both macrophage-tropic (CCR5 preferring) and lymphotropic (CXCR4 preferring) phenotypes. Preliminary support for this hypothesis has come from Petito *et al* (Petito *et al*, 1999; Chen *et al*, 2000) who recently reported that viral sequences in choroid plexus represent an admixture of variants resembling sequences detected in both brain and blood.

The extent to which choroid plexus infection may contribute to the viral burden in CSF is currently unclear. However, the HLA-DR+ cells described by Hanly and Petito (1998) were observed on both the stromal and apical surfaces of the choroidal epithelium, with occasional processes extending between adjacent epithelial cells. Thus, the distribution of these putative DCs on either side of the blood-CSF barrier suggests that they could be capable of releasing progeny virions directly into the CSF. Alternatively, CSF penetration of virus may take place via trafficking of locally infected macrophages or lymphocytes across the blood-CSF barrier, analogous to the "Trojan Horse" mechanism proposed to occur at the blood–brain barrier. Increased expression of MIP-1α, MIP-1β, and RANTES reported in the CSF of HIV-1-infected patients (Kelder *et al*, 1998; Letendre *et al*, 1999) might further encourage replenishment of the macrophage population by circulating monocyte precursor cells. CSF viremia during the early stages of infection often correlates with CSF mononuclear cell counts (McArthur *et al*, 1989; Ellis *et al*, 1997; Martin *et al*, 1998; Eggers *et al*, 1999; Gisslen *et al*, 1999), which may be elevated even though the albumin index and lack of red blood cells reveal that the blood–brain and blood-CSF barriers remain intact (Ellis *et al*, 1997). Although to date there has been no direct demonstration of immune cell trafficking in the choroid plexus, the potential for cell migration to occur is underscored by observations of inflammatory cell infiltrates within the choroid plexus stroma of lentivirus-infected hosts (Dean *et al*, 1993; Beebe *et al*, 1994; Falangola *et al*, 1995; Czub *et al*, 1996a, 1996b; Hanly and Petito, 1998); and, the increased adhesion molecule expression on the choroidal epithelium that is often associated with CNS inflammation (Marker *et al*, 1995; Steffen *et al*, 1996; Wolburg *et al*, 1999). In addition, Liu *et al* (2000) recently detected a cluster of identical sequences in HIV-1 isolates recovered from choroid plexus, meninges, and lymph nodes. Phylogenetic analysis of these clones suggested the movement of virions or infected cells from choroid plexus into the CSF and then lymph nodes. These sequences differed from those in the deep white matter suggesting an independent mode of trafficking at the blood-CSF barrier.

The theoretical model of choroid plexus infection that emerges from these observations suggests far more dynamic immune cell interactions and transfer of infectious virus than has previously been proposed. The density of macrophages in the stroma of the choroid plexus between the vasculature and epithelium provides a highly concentrated and active area for virus capture, infection, and transfer. Our findings, using an *in vitro* system, has demonstrated that FIV can establish a low-grade productive infection of choroid plexus macrophages and perhaps DC-like cells that may be amplified upon exposure to CD4+ T-lymphocytes. Future investigations are required to fully determine the precise phenotype of the putative choroid plexus dendritic cells, as well as the extent to which the choroid plexus may seed the CSF with various viral isolates. In this regard, the FIV model should continue to serve as a valuable tool that allows both *in vivo* and *in vitro* studies to be conducted in parallel.

Materials and methods

Experimental animals

Specific pathogen-free (SPF) cats were obtained from Liberty Labs (Liberty Corner, NJ), maintained and bred at the North Carolina State University College of Veterinary Medicine (NCSU-CVM). Additional fetal tissue was obtained from random source cats spayed at the NCSU-CVM clinics.

Primary cultures of feline choroid plexus

Fetuses were removed by cesarean section at approximately 25 to 40 days gestation. Brains were removed from the cranium and rinsed 3× in fresh sterile Hanks' balanced salt solution (HBSS). Choroid plexus was dissected from all four ventricles and rinsed in HBSS. Tissue explants were pooled and minced with sterile forceps, and the resulting cell suspension was seeded uniformly onto poly-D-lysine-coated (0.1 mg/ml) glass coverslips in each well of a 24-well plate. Cells were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 20 μg/ml gentamycin (complete medium) in a 5% CO_2 incubator at 35 to 36°C. Macrophages quickly attached to the coverslip, whereas clumps of choroidal epithelium remained only loosely adherent.

The cultures consisted initially of a mixed population of choroid plexus macrophages and epithelial cells, with some fibroblasts and endothelial cells. For the first 8 days *in vitro*, cultures were vigorously washed 3X in HBSS every two days and fed with complete medium. This procedure guaranteed that small amounts of nonadherent leukocytes released from the choroid plexus vasculature were not present *in vitro* before beginning the infection studies. These culture conditions favored growth of choroid plexus macrophages but not epithelial cells, which do not firmly attach to the poly-D-lysine substrate. After 8 days *in vitro*, choroid plexus cultures were typically

enriched in macrophages at densities ranging from approximately 50 to 170 cells/mm^2. Fibroblasts, endothelial cells, and small amounts of epithelium were present as well. Prior to the infection studies, choroid plexus cultures were maintained in complete medium supplemented with recombinant human CD40-ligand trimer (100 ng/ml; generously provided by Immunex Corporation; Seattle, WA) for 24 h to promote the differentiation of the macrophages into dendritic-like cells.

Source of FIV

Choroid plexus cultures were inoculated with either an infectious molecular clone of the NCSU$_1$ strain of FIV (FIV-NCSU$_1$), prepared as previously described (Yang *et al*, 1996), FIV-MD, (provided by Dr. Mike Podell, Ohio State University), or FIV-PPR (provided by Drs. John Elder and Tom Phillips, Scripps Research Institute). All strains were grown in a feline interleukin-2-dependent, CD4+ T-lymphocyte cell line, FCD4-E, established in our laboratory as previously described (English *et al*, 1993).

FIV inoculation of choroid plexus and coculture with T-lymphocytes

After 7 to 8 days *in vitro*, choroid plexus cultures were inoculated with FIV at an approximate multiplicity of infection (M.O.I.) of 0.1, based on estimates of the total number of macrophages *in vitro*. Uninfected control cultures were inoculated with vehicle (complete medium). Cells were exposed to virus for 48 h, washed 3× in HBSS, then fed with complete medium. Tissue culture supernatants were collected on days 5, 8, and 11 postinoculation, centrifuged at 1000× *g* for 15 min to remove any suspended cells, and stored at −80°C for subsequent p24 antigen analysis. Immediately after collection of medium on day 11, choroid plexus cultures were seeded with the CD4+ feline T-lymphocyte cell line, FCD4-E, at an approximate density of 5×10^3 cells/cm^2. Culture medium was collected again at 6 and 12 days following addition of FCD4-E cells. Choroid plexus inoculated with FIV was also cocultured with FCD4-E cells that had been irradiated (10000 rads) to determine whether exposure to T-lymphocyte membranes might be sufficient to stimulate increased production of virus.

Multiple controls were performed to verify that nonspecific binding of virus *in vitro* did not contribute to infection of T-lymphocytes. Primary cultures of dissociated rat cortex, which are not permissive to FIV infection, were inoculated with virus, cocultured with lymphocytes, and sampled for p24 antigen as outlined previously. In addition, feline choroid plexus cultures were exposed to FIV for 48 h, washed 3× in HBSS, and incubated in trypsin (10 μg/ml in serum-free DMEM, 37°C) to digest any remaining cell-free virions. After 20 minutes, the enzyme was inactivated by washing cultures 3× and feeding with medium containing serum. Collection of supernatants and coculture with lymphocytes proceeded by the same protocol. To verify that the trypsin digestion protocol was sufficient to abolish infectivity, FCD4-E cells, which are highly susceptible to FIV infection, were inoculated at an M.O.I. of 0.1 with an FIV-NCSU$_1$ stock that had been pre-treated with trypsin (10 μg/ml, 20 min at 37°). Medium from inoculated FCD4-E cell cultures was sampled for p24 analysis on days 5, 8, 11, 17, and 23 postinoculation.

Polymerase chain reaction (PCR) amplification of FIV provirus

Choroid plexus cells were harvested at 2 weeks postinoculation with FIV-NCSU$_1$. DNA was extracted with DNAZol (Life Technologies), precipitated and washed in 100% ethanol, resuspended in PCR buffer, and stored at −80°C. FIV provirus was detected in infected cells via nested PCR amplification with conserved gag-specific primers. Primers FIV-7 (5′-TGACGGTGTCTACTGCTGCT) and FIV-8 (5′-CACACTGGTCCTGATCCTTTT) amplified an 838-base pair (bp) segment, while primers FIV-1 (5′-CCACAATATGTAGCACTTGACC) and FIV-2 (5′-GGGTACTTTCTGGCTTAAG GTG) amplified a 582-bp segment included within the region delimited by FIV-7 and FIV-8. PCR amplification was performed in a total reaction volume of 25 μl that contained 1 μg of template DNA, 0.025 μg of each primer, 25 μM dNTP, 1 × PCR buffer + 1.5 mM $MgCl_2$, and 0.01 units/μl Taq polymerase (Life Technologies). The amplification protocol of 95°C for 45 s, 59°C for 45 s, and 72°C for 45 s was repeated for 35 cycles, followed by a 5-min, 72°C extension. These products then served as templates for nested PCR amplification with the internal primers FIV-1 and FIV-2 in a reaction mixture containing additional dNTP, buffer +1.5 mM $MgCl_2$, primers and Taq polymerase at a final volume of 25 μl. After an additional 25 cycles of amplification, final products were identified by electrophoresis on a 1.2% agarose gel and visualized by staining with 10 μg/ml ethidium bromide.

ELISA for FIV p24 core antigen

Cell-free tissue culture supernatants were assayed for the viral core antigen by a commercially available ELISA (Idexx Laboratories, Westbrook, ME), using a monoclonal antibody specific to the FIV p24 protein. Then, 100-μl aliquots of culture supernatants were assayed according to the manufacturer's protocol. Products were detected via horseradish peroxidase with tetramethylbenzidine and quantified by spectrophotometry according to the manufacturer's instructions

Immunohistochemistry for FIV p24 core antigen

Infected choroid plexus cells were identified *in vitro* by immunohistochemistry (IHC) for the FIV p24 core protein. Because cocultures of choroid plexus and

FCD4-E cells included a mixture of adherent and nonadherent cells, an equal volume of 4% paraformaldehyde was added directly to the culture medium to fix and attach cells to the poly-D-lysine substrate. After 60 min, coverslips were washed gently in phosphate-buffered saline (PBS; 0.01 M, pH = 7.4) and coated with gelatin (0.5% solution) to help preserve the loosely adherent cells. Endogenous peroxidase activity was quenched by incubation in hydrogen peroxide (0.6% in methanol, 30 min), and nonspecific binding sites were saturated by preincubation in 5% normal goat serum supplemented with 2% bovine serum albumin (BSA) and 0.1% sodium azide in PBS (IHC buffer). Cultures were incubated in either primary antibody (mouse-anti-p24, Custom Monoclonals International; 2.5 μg/ml IgG in IHC buffer;) or pre-immune serum (normal mouse serum, diluted 1:20000 in IHC buffer to achieve equivalent concentrations of IgG). To avoid nonspecific binding of the secondary antibody to F_c receptors that may be expressed within the choroid plexus, we used a biotinylated secondary antibody containing a deletion of the F_c region (AffiniPure Fab fragment, goat-antimouse IgG; Jackson Immunoresearch Laboratories; West Grove, PA). Products were detected via ABC method (Vector Laboratories, Burlingame, CA) using diaminobenzidine as chromagen with cobalt chloride and nickel chloride enhancement.

Immunohistochemistry data analysis

Cells were viewed under oil immersion at a magnification of 561× on an Olympus IMT-2 inverted microscope. Digital images were captured and stored by a Metamorph Imaging System (Universal Imaging Corporation, West Chester, PA). For densitometric analysis, digital images were enlarged to a final magnification of 2246×. Cells were individually traced, an average gray scale value was measured within the defined region, and an average optical density was calculated for each cell. A frequency distribution of density values for all cells in each condition was generated and changes in the densities analyzed using a chi-squared test.

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