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Effects of feline immunodeficiency virus on astrocyte glutamate uptake: Implications for lentivirus-induced central nervous system diseases

(HIV-1/glucose)

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ABSTRACT Feline immunodeficiency virus (FIV) is a lentivirus of domestic cats that causes a spectrum of diseases remarkably similar to AIDS in HIV-infected humans. As part of this spectrum, both HIV-1 and FIV induce neurologic disorders. Because astrocytes are essential in maintaining the homeostasis of the central nervous system, we analyzed FIV for the ability to infect feline astrocytes. Through immunocytochemistry and reverse transcriptase activity, it was demonstrated that two molecular clones of FIV (FIV-34TF10 and FIV-PPR) produce a chronic low level productive infection of feline astrocyte cultures. To investigate the consequences of this infection, selected astrocyte functions were examined. Infection with FIV-34TF10 significantly decreased the ability of astrocytes to scavenge extracellular glutamate (with a peak inhibition of 74%). The effects of the infection did not appear to be a result of toxicity but rather were more selective in nature because the glucose uptake function of the infected astrocyte cultures was not altered. Our data demonstrate that FIV productively infected, at a low level, feline astrocyte cultures, and as a consequence of this infection, an important astroglial function was altered. These findings suggest that a chronic low grade infection of astrocytes may impair the ability of these cells to maintain homeostasis of the central nervous system that, in turn, may contribute to a neurodegenerative disease process that is often associated with lentivirus infections.

Two members of the lentivirus family, HIV type-1 (HIV-1) and feline immunodeficiency virus (FIV), share many structural and biochemical properties (1). Clinically, they induce remarkably similar syndromes, including severe immunosuppression and neurologic dysfunction (2). In clinical studies, behavioral abnormalities of FIV-infected cats suggested that cortical and subcortical structures in the brain had become infected (3); subsequently, FIV was recovered from the cerebrospinal fluids and brain tissues of naturally and experimentally infected cats (4, 5). FIV and HIV-1 produce similar neurologic deficits in their respective hosts, including: anisocoria, alterations in pupillary reflexes, delayed auditory and visual evoked potentials, decreased nerve conduction velocities, and abnormal sleep architectures (6–10). The major difference between FIV- and HIV-1-induced central nervous system (CNS) disease is that FIV-infected cats have relatively few multinucleated giant cells at necropsy (11). Thus, there are many similarities between these two viruses, and FIV has been shown to be a good model for studying the effects of lentivirus on the CNS.

Although both HIV-1 and FIV cause neurological disease and alter CNS function, neither virus has been demonstrated to infect neurons (12–14). Thus, it is likely that the effects of lentiviruses on neurons are indirect (12, 14). Although HIV-1 infection of CNS once was thought to be limited to microglia (12), there is increasing evidence that astrocytes are also a target for infection (15–27). The level of HIV-1 replication in astrocytes is low (21, 23, 27, 28), but a persistent productive infection clearly develops (20, 29).

Astrocytes by far are the most common cell type of the brain and are important in maintaining the neuronal microenvironment in the CNS (30, 31). One of the most important functions of astrocytes is to regulate the level of extracellular glutamate, a major excitatory neurotransmitter (32). Glutamate accumulates as a consequence of neuronal activity (32). Excessive levels of extracellular glutamate often result in neuron toxicity and death (32). Thus, the astrocyte function of controlling extracellular glutamate levels in the brain is essential to maintaining the health of the neuron. Another important astrocyte function is the regulation of energy stores for the brain (33). Astrocytes, to a large extent, regulate the level of extracellular glucose and also maintain large glycogen stores (33). Neurons depend on astrocytes to supply a ready source of energy to meet their high metabolic demands (34).

To examine a potential mechanism of lentivirus-induced neurologic effects, we exposed cortical astrocytes from specific pathogen-free cats to two variants of FIV. The results confirmed that these cells were susceptible to infection by FIV and that infection by one variant severely altered glutamate uptake, an essential astrocyte function.

MATERIALS AND METHODS

Cells and Viruses. For these studies, we used two previously described infectious molecular clones of FIV, 34TF10, and peeper (PPR) (10, 35, 36). FIV-34TF10 was established from a tissue culture-adapted strain of the Petaluma isolate of FIV. This viral strain infects the Crandell feline kidney cells (CrFK) and G355–5 cell lines but replicates less efficiently on feline peripheral blood leukocytes (35). FIV-PPR is a molecular clone that was established from the San Diego strain of FIV and, like the parent isolate, has a more typical lentivirus tropism of macrophages and lymphocytes (35). FIV-PPR replicates well in cats and causes a neurologic as well as a immunological form of the disease, whereas FIV-34TF10 replicates poorly *in vivo* and produces little disease (10, 35).

A viral stock of FIV-34TF10 was prepared by introducing 10 μg of viral DNA into CrFK cells by electroporation (36). Viral

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Abbreviations: CrFK, Crandell feline kidney cells; RT, reverse transcriptase; GFAP, glial fibrillary acidic protein; MDCK, Madin Darby canine kidney cell; [^{3}H]2DG, 2-deoxy-D-[^{3}H]glucose; PPR, peeper.

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replication was monitored by reverse transcriptase (RT) assay, as described (35). A stock of FIV-PPR was produced in a similar manner, except that cocultivation with feline peripheral blood lymphocytes was performed 1 day after transfection into CrFK to allow efficient FIV-PPR production. Madin Darby canine kidney cell is an adherent cell line (MDCK, ATCC CCl-34) that is resistant to FIV infection. MCH5–4 is a feline T lymphocyte cell line (37) that supports FIV-PPR replication.

Enriched Astrocyte Cultures. Primary cultures of cerebral cortical astrocytes were prepared from fetuses at the 45th day of gestation or from 2-day-old offspring of specific pathogen-free cats as described (38) with some modification (39). In brief, forebrains were removed aseptically from the skulls; the meninges were excised carefully under a dissecting microscope, and neocortices were dissected. The cells were dissociated, without trypsin, by passage through needles of decreasing gauges (16G1, 19G1, and 25G1) 2–3 times with a 10-ml syringe. The cells were seeded at a density of 10^5 cells/cm^2 on 12-well tissue culture plates (Costar) in DMEM (BioWhittaker) containing 10% fetal bovine serum (BioWhittaker) and 25 mM glucose and then incubated at 37°C in an atmosphere containing 5% CO_2 at 95% humidity. The culture medium was renewed 3 to 4 days after seeding and subsequently twice per week. These conditions yielded astrocyte cultures containing >92% glial fibrillary acidic protein (GFAP) immunoreactive cells (39). Cellular cultures also were stained for nonspecific esterase to quantify the number of microglial cells (40).

Infection of Astrocytes. Cerebral cortical astrocytes and MDCK control cells were seeded in 12-well plates (Costar) to obtain 50–60% confluence on the day of infection. Then, FIV-34TF10 or FIV-PPR virus was added for each replicate experiment at a multiplicity of infection varying from 5 to 20 for PPR and 0.5 to 2 for 34TF10. After 1 day of incubation, the cultures were washed in serum-free media and trypsinized to remove the residual infecting viral particles. Identical cultures were then placed into two experimental protocols. One set of the cultures was resuspended and maintained in DMEM 10% fetal bovine serum for weekly monitoring of RT activity and was used in the astrocyte functional assays. The other set of cultures was cocultivated with the indicator cell lines of either CrFK (for FIV-34TF10 infections) or MCH5–4 cells (for FIV-PPR infections) to determine whether infected astrocyte cultures produced infectious virus. To determine whether the washing and trypsinization was effective in removing the residual infecting virus, a similar infection and coculture protocol was applied to MDCK cells, a cell line that is resistant to FIV infection.

Immunocytochemistry. For immunostaining experiments, feline cortical astrocytes were seeded on slides (previously polylysine-coated; Nunc) and infected as described above. Three days after infection, cells were washed in cold PBS (BioWhittaker) and fixed in methanol. Polyclonal antibodies directed against GFAP, a specific astrocyte marker (Sigma) and FIV-positive serum determined by ELISA (FIV antibody test kit, Idexx Laboratories, Westbrook, ME) were used for the primary incubation at room temperature for 30 min. Next, the cells were washed in PBS and incubated with either a donkey anti-rabbit lissamine rhodamine conjugate (Jackson ImmunoResearch) or a fluoresceinated goat anti-cat antibody (Sigma). Slides were observed under a Nikon microscope and photographed.

RT Assay. Virus-containing supernatants were centrifuged at 230,000 × *g* for 45 min at 22°C. The tubes were drained and swabbed to remove any residual medium, and then the viral pellets were resuspended thoroughly in 100-μl of reaction mix (20 mM DTT/40 mM Tris/360 mM NaCl/2% Nonidet P-40). The samples were freeze-thawed two times in a dry ice/ethanol bath to disrupt virions, and then 25 μl was mixed with an equal volume of buffer containing 40 mM Tris·HCl (pH 8.1), 60 mM NaCl, 0.02 units of polyriboadenosine:polydeoxythymidine (Pharmacia), 0.625 μCi (1 Ci = 37 GBq) of [^{3}H]dTTP (80 Ci/mmol, NEN), and either 160 mM $MgCl_2$ or 1.2 mM $MnCl_2$ (negative control). After incubation for 1 hr and 30 min at 37°C, the samples (in quadruplicate) were spotted on Whatman DE81 filters and then were washed first with 0.1 M sodium pyrophosphate and second with 0.3 M ammonium formate. The filters then were washed with 95% ethanol, dried under a heat lamp, and examined for radioactivity by liquid scintillation counting (Packard).

Glucose Uptake Assay. Uptake experiments were conducted weekly after infection, as detailed in our previous study (39). In brief, after the medium was removed, cells were incubated for 3 hr in 0.5 ml of serum-free DMEM containing 5 mM instead of 25 mM glucose (DMEM5) at 37°C in an atmosphere containing 5% CO_2 at 95% humidity. Afterward, 0.5 ml of fresh DMEM containing 2-deoxy-D-[^{3}H]glucose ([^{3}H]2DG) (final concentration, 48 nM) was added for an additional 20-min incubation. Uptake was terminated by aspirating the uptake solution and washing the cells three times with 2 ml of ice-cold PBS. Astrocytes then were lysed by adding 0.5 ml of 10 mM NaOH containing 0.1% Triton X-100, and a 300-μl portion was assayed for ^{3}H by liquid scintillation counting. The protein content was measured by the method of Bradford (41) in 100 μl of the remaining lysate. [^{3}H]2DG uptake was expressed in femtomole per milligram protein.

Glutamate Uptake Assay. The uptake of [^{3}H]glutamate was determined by the method described by Volterra and coworkers (42). In brief, after infection and supernatant collection, the medium was replaced by 0.5 ml of fresh medium containing 50 μM glutamate and 18.5 kBq (9.25 pmol) of [^{3}H]glutamate. Uptake was terminated 15 min later by removing the supernatant and washing the cells three times with 2 ml of ice cold PBS containing 5 mM glutamate. Astrocytes were then lysed by 0.5 ml of 10 mM NaOH containing 0.1% Triton X-100, and a 300-μl portion was assayed for ^{3}H by liquid scintillation counting. The protein content was measured (41) in 100 μl of the remaining lysate. Uptake was expressed in femtomole per milligram protein.

RESULTS

Preparation of Enriched Astrocyte Cultures. As Fig.1*A* illustrates, cultures of feline astrocytes became confluent after 4–5 weeks, showing a highly homogeneous morphology with dense cell bodies and multiple processes. After reaching confluence, the cells stopped growing and kept their morphologic status for as long as 3–4 mo (not shown). To confirm that the cells were astrocytes, we added an anti-GFAP antibody and found that >92% were GFAP-positive (Fig. 1*B*) and <2.5% were microglial cells as determined by nonspecific esterase staining (data not shown).

Feline Cortical Astrocytes Are Susceptible to FIV Infection *in Vitro*. Feline cortical astrocytes, at 50–60% confluence, were infected with either FIV-34TF10 or FIV-PPR. At 3 days postinfection, viral antigens were detected in both cultures. That is, FIV-positive serum reacted with the astrocytes infected with either 34TF10 or PPR (Fig. 2 *A* and *C*). All of the FIV-positive cells also were GFAP-positive (Fig. 2 *B* and *D*). However, no specific staining for FIV antigens was observed after adding FIV-positive serum to mock-infected astrocytes nor when combining FIV-negative serum with FIV-infected astrocytes (data not shown). After 3 days of viral exposure, only a few cells appeared to be infected but all of those infected had the features of astrocytes (GFAP-positive).

A productive infection of feline astrocytes by FIV-34TF10 was detectable after 3 days and increased with time, becoming statistically significant at 15 days and reaching a peak after 29 days (Fig. 3*a*). In contrast, throughout this period, astrocytes infected with the FIV-PPR isolate produced only low levels of RT activity even when the multiplicity of infection was 10 times

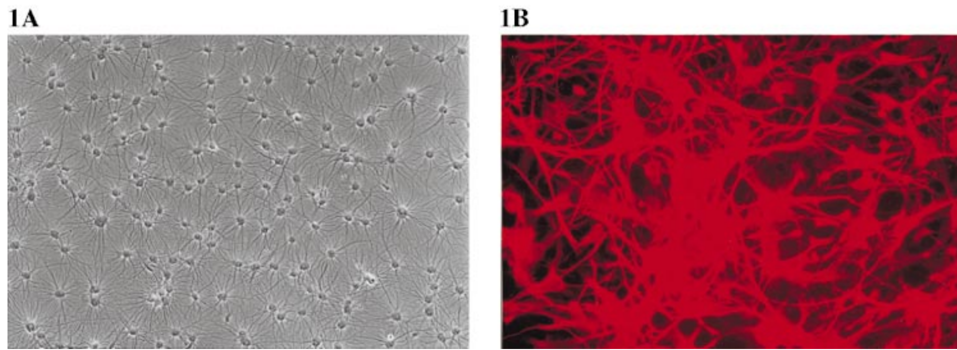

FIG. 1. Morphology and characterization of cultured feline astrocytes. (*A*) Phase–contrast micrograph of primary astrocyte cultures maintained with DMEM supplemented with 10% fetal bovine serum for 6 weeks (×100). (*B*) Cultured astrocytes stained with rabbit anti-bovine GFAP and visualized by immunofluorescence with donkey anti-rabbit lissamine rhodamine B sulfonyl chloride-conjugated (×200).

the level used for 34TF10 (Fig. 3*b*). However, when the FIV-PPR-infected astrocyte cultures were cocultivated with a FIV-PPR-susceptible feline T cell line (MCH5–4), statistically significant RT activity was detected at 15 days postinfection and continued to increase (Fig. 3*c*), indicating that FIV-PPR-infected astrocyte cultures produced a low level of infectious virus. In contrast, coculture of the FIV-PPR-infected MDCK cells (a FIV-resistant cell line) with MCH5–4 cells yielded only background levels of RT activity at 15 days, and the weak response at 20 days postinfection was attributed to residual viral particles left after washing and trypsinization. Cocultivation of the FIV-34TF10-infected astrocyte cultures with CrFK cells (an indicator cell line) demonstrated that infectious virions were produced by a FIV-34TF10 clone (data not shown).

Glucose Uptake of FIV-Infected Astrocytes. Uptake of ^{3}[H]2DG was used to assess glucose uptake of astrocytes. In uninfected cultures, the average value of ^{3}[H]2DG uptake was 691 ± 49 fmol/mg protein in 16 independent assays, closely resembling the range (299–630 fmol/mg protein) in mouse astrocytes (43). As Fig. 4*a* depicts, [^{3}H]2DG uptake changed little during the first week of infection by 34TF10, but 2 weeks after infection, [^{3}H]2DG uptake increased 61% before returning to the basal level at 3 weeks of infection. However, this increase in [^{3}H]2DG uptake was not statistically significant. The FIV-PPR virus also did not significantly alter [^{3}H]2DG uptake throughout the 4-week test period (Fig. 4*b*).

Glutamate Uptake of FIV-Infected Astrocytes. Uptake of [^{3}H]glutamate was used to assess the ability of astrocytes to buffer the glutamate in extracellular spaces. In 16 independent experiments, the basal rate of [^{3}H]glutamate uptake into astrocytes was an average value of 7.5 ± 1.1 nmol/mg protein/min, resembling that for mouse astrocytes (4.48 ± 0.26 nmol/mg protein/min) (44). After 1 week of infection by FIV-34TF10, the feline astrocytes underwent an ≈20% reduction of [^{3}H]glutamate uptake. Thereafter, 65%, 41%, and 74% inhibition of [^{3}H]glutamate uptake was observed after 14, 21, and 28 days, respectively (Fig. 5*a*). These results were statistically significant for the 14- and 28-day time points, to the $P < 0.001$ level (Fig. 5*a*). In comparison, FIV/PPR induced a

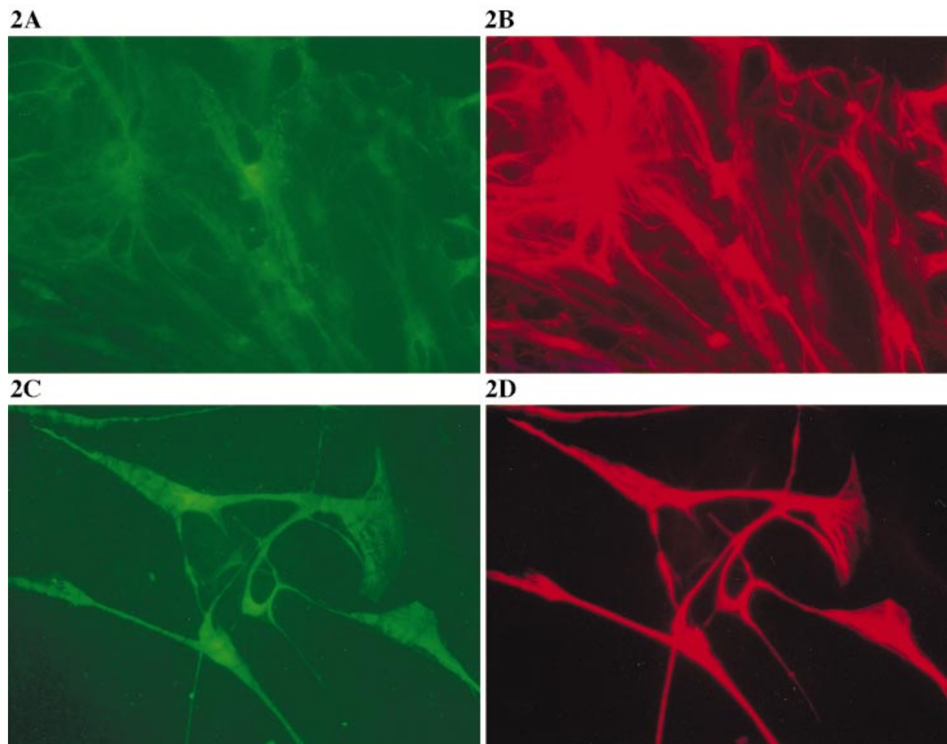

FIG. 2. Detection of GFAP and viral proteins in FIV-infected feline astrocytes. Three days after the infection, the cultures were double-stained for FIV and GFAP. (*A*) Viral protein staining of FIV-34TF10-infected cells; (*B*) GFAP staining of FIV-34TF10-infected cells (same field as *A*). (*C*) Viral protein staining of FIV-PPR-infected cells. (*D*) GFAP staining of FIV-PPR-infected cells (same field as *C*) (×400).

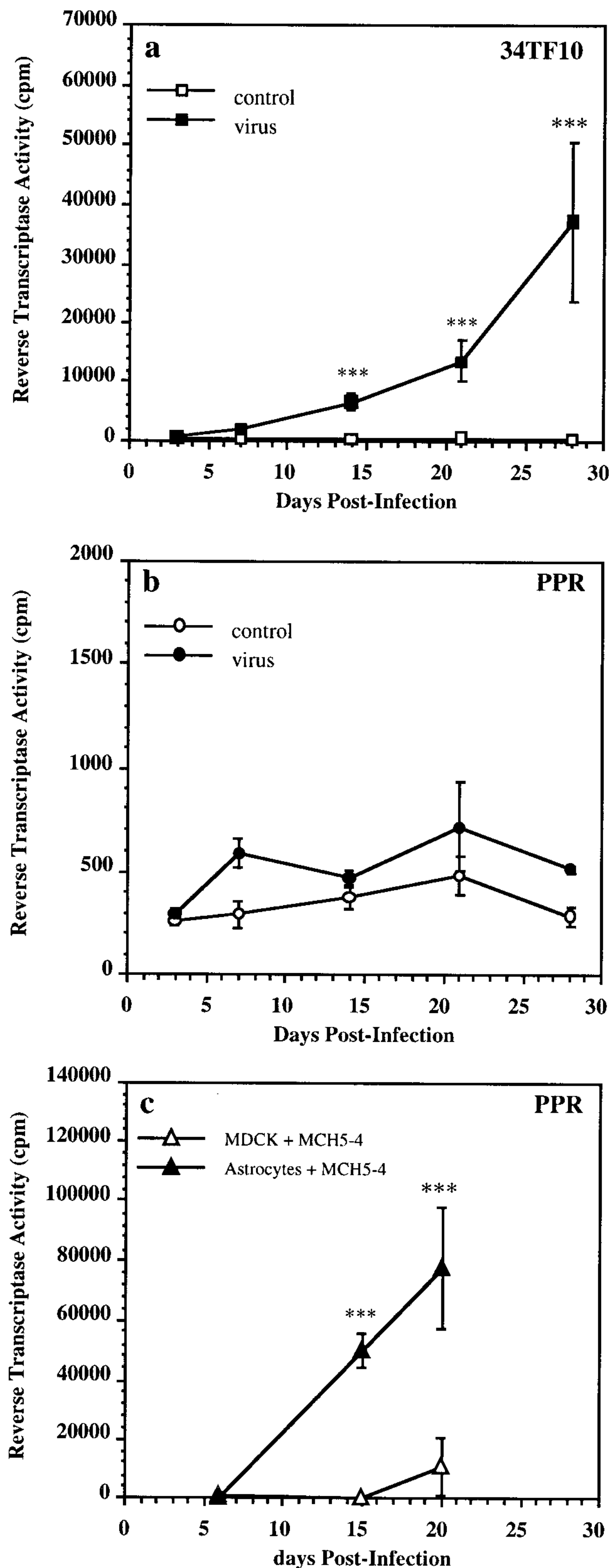

FIG. 3. RT activity in culture supernatant of feline cortical astrocytes after infection with the molecular clones of FIV. RT activity from the supernatants of feline astrocyte cultures, which were infected with either FIV-34TF10 (*a*) or FIV-PPR (*b*), was plotted over time and compared with uninfected cultures. (*c*) FIV-PPR-infected feline astrocytes were cocultivated with an indicator cell line, MCH5–4, or an FIV-restricted cell line, MDCK. RT activity from the cocultured supernatants was plotted over time. Results are expressed as mean cpm ± standard error of the mean. Results were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

weak decrease of [^{3}H]glutamate uptake during 1 mo postinfection (Fig. 5*b*), but a significant difference was never found with this viral clone.

DISCUSSION

This present study is the first demonstration of a lentiviral infection altering the astrocyte function of glutamate uptake. This function is essential in maintaining the homeostasis of the CNS. Because it is held widely that lentiviruses do not infect neurons, the disruption of this astroglial function indicates a potential indirect mechanism of lentivirus-induced CNS dysfunction.

The suppressive effects of FIV on glutamate uptake are particularly important because there is a large body of evidence supporting the excitotoxicity theory of lentivirus-induced CNS disease in which the extracellular concentration of glutamate may play an essential role in the developing neurotoxicity (45–48).

The findings of this present study support the results of two recent reports on HIV-1 that showed that the exogenous addition of glycoprotein 120 to astrocyte cultures impeded the ability of these cells to remove glutamate from the extracellular fluid (49, 50). However, neither of these earlier studies looked at the effects of infectious virus on glutamate uptake. Because it is clear, both *in vitro* (15, 16, 19, 22, 24, 27, 29) and *in vivo* (17, 20, 21, 23, 25, 26, 28), that astrocytes are a target for lentivirus replication, we elected to examine the effects of a lentivirus infection on the ability of the astrocytes to scavenge glutamate from the extracellular fluid and found that, at 2 weeks postinfection, the FIV-34TF10 clone significantly suppressed glutamate uptake. This level of suppression (50–60%) was maintained throughout the remainder of the study (Fig. 5*a*). The other FIV clone that was examined in this study (FIV-PPR) did not significantly inhibit glutamate uptake, although a similar mild inhibitory effect was detected with this clone (Fig. 5*b*).

The difference in these two clones in their ability to affect astrocyte function is interesting because the FIV-PPR clone causes a CNS dysfunction in infected cats whereas the FIV-34TF10 clone does not. FIV-34TF10 is a laboratory-adapted clone that replicates efficiently *in vitro*. Thus, the difference between these two clones on astrocyte function might reflect their relative ability to replicate in astrocytes *in vitro* (35). Although FIV-PPR astrocyte replication was demonstrated by immunocytochemistry and RT assay, the level of this replication was markedly less than the FIV-34TF10 clone. Given the slow onset on CNS dysfunction after lentivirus infections, moderate alterations in astrocyte function could be responsible for the slow progressive CNS disease that is typical of these infections. Thus, the effects of the FIV-PPR clone may be a more typical consequence of an FIV infection on feline astrocyte function. Although the FIV-34TF10 effects might be viewed as a heightened pathological result due to the more efficient replication in the permissive *in vitro* environment, it also may effectively demonstrate a possible *in vivo* FIV pathogenic potential. The true *in vivo* relevance of these findings needs further study. However, the mere demonstration that a strain of FIV was able to alter astrocyte glutamate uptake *in vitro* could be viewed to mean that, under certain conditions, similar mechanisms might be operating in the FIV-infected animal.

Using immunocytochemistry in conjunction with an RT assay, we show here that both clones of FIV productively infected feline astrocyte cultures, albeit at a low level of replication. These results are similar to the reported findings with HIV-1 (16, 17, 19–23, 25–29) and previous studies on FIV (15, 24). Thus, although macrophages appear to harbor a more productive infection, the astrocyte, as the most numerous cell type in the brain, may serve as an important lentivirus reser-

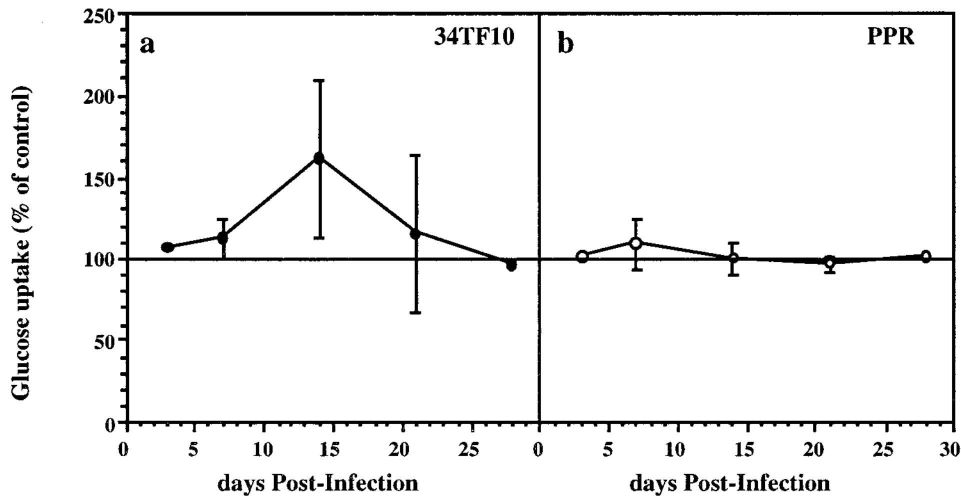

FIG. 4. Effects of FIV infection on astrocyte glucose uptake. At the indicated time points with either FIV-34TF10 (*a*) or FIV-PPR (*b*), the ability of the feline astrocyte cultures to remove [^{3}H]2DG from the culture supernatant was determined, as described in *Materials and Methods*. Results are expressed as the ratio of [^{3}H]glucose uptake of infected cultures over the value of the control cultures, ± SEM. Results were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

voir, particularly because many of the current lentivirus therapeutics do not cross the blood–brain barrier.

It is also interesting to note that, in this present study, glucose uptake of FIV-34TF10-infected astrocyte cultures was not impaired (if anything, an increase in this activity was suggested) (Fig. 4*a*). Because normal glucose uptake occurred concurrently with the significant decrease in glutamate uptake, it is unlikely that a generalized cell toxic effect can explain these results but rather the virus infection resulted in selective alteration to a specific astrocyte function.

Others (24) also have demonstrated that FIV infections can affect astrocyte functions because FIV-infected astroglia displayed a significant delay in achieving peak Ca^{2+} levels after ionophore application and a decrease in the amount of Ca^{2+} released from intracellular stores. Furthermore, plasma membrane lipid mobility increased and glutathione decreased (24). These results combined with the results of the present study indicate that FIV infection has profound effects on astrocyte function.

It has been proposed that the effects of lentivirus on astrocyte function might be indirect, through the virus or its proteins acting on microglia, which in turn produce a variety of products (e.g., arachidonic acid, tumor necrosis factor-α, and reactive oxygen intermediates) that are known to suppress the ability of the astrocyte to scavenge glutamate from the environment (44, 49, 51–56). Certainly, such indirect mechanisms are likely to be operating and contributing to the pathology. However, with lentiviruses infecting astrocytes both *in vivo* and *in vitro*, direct effects of the lentivirus infection on astrocyte function cannot be discounted because even a restricted virus infection can substantially alter cellular functions (57). It is likely that both direct and indirect effects of lentivirus infections are combining to alter normal astrocyte function (58).

Because rodent astrocytes are commonly used for studies of this type, a comparison between the rodent and the feline astrocytes is appropriate. The present results reveal that ranges of both glutamate uptake and glucose uptake by feline astrocytes closely resembled the ranges found in our previous studies with mouse astrocytes (43, 44). This similarity indicates that astrocytes from mice and cats have the same capacity to buffer extracellular glutamate and the same rate of turnover

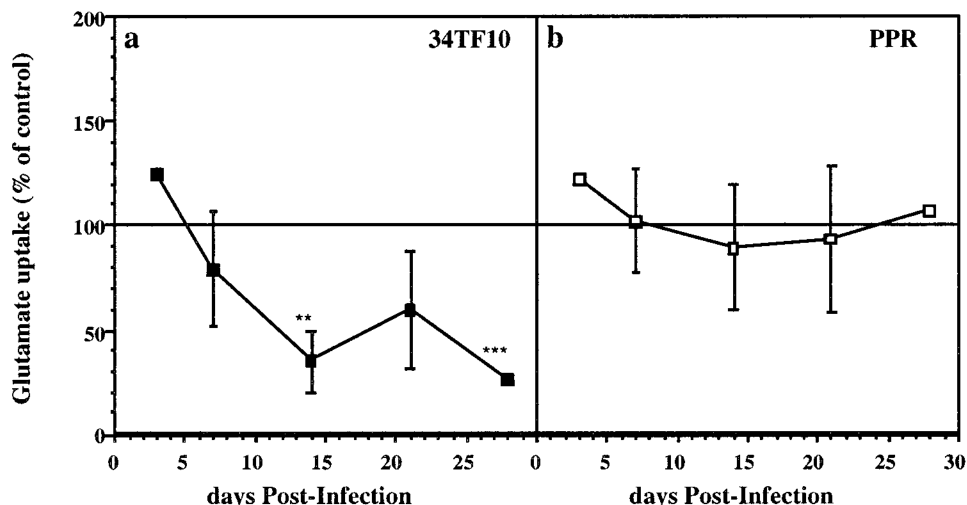

FIG. 5. Effects of FIV infection on feline astrocyte glutamate uptake. At the indicated time points, after infection with either (*a*) FIV-34TF10 or (*b*) FIV-PPR virus, the [^{3}H]glutamate uptake assay was performed, as described in *Materials and Methods*. Results are expressed as the ratio of [^{3}H]glutamate uptake of infected cultures over the value of the control cultures, ± SEM. Results were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

for its energy substrate. However, astrocytes from the different animal species vary morphologically. By phase–contrast microscopy, murine astrocytes were fibroblast-like without visible processes (data not shown), whereas feline astrocytes had a more dense cell body and, apparently, multiple processes (Fig. 1*A*). Thus, our results clearly demonstrate that cultured feline astrocytes are a suitable model for the studies of astrocyte functions.

The results of this study demonstrate that FIV-34TF10-infected astrocyte cultures developed a decreased ability to scavenge glutamate. This astrocyte function is essential in maintaining the homeostasis of the CNS. The disruption of this astroglia function may contribute to lentivirus-induced CNS disease and supports the hypothesis of glutamate toxicity as a factor in AIDS dementia (14).

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