Journal of NeuroVirology (2000) 6, Suppl 1, S90–S94
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Stages of restricted HIV-1 infection in astrocyte cultures derived from human fetal brain tissue

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The predominant cell types infected by HIV-1 in AIDS associated encephalopathy are cells of the macrophage/microglial lineage. There has been consistent evidence, however, that astrocytes also become infected although not at the same frequency or level of multiplication as microglial cells. HIV-1 antigens and/or nucleic acid have been identified in astrocytes in brain autopsy tissue from both adult and pediatric AIDS cases. In cell cultures, HIV-1 infection of astrocytes results in an initial productive but non-cytopathogenic infection that diminishes to a viral persistence or latent state. Understanding the nature of HIV-1 infection of astrocytes, which represents the largest population of cells in the brain, will contribute to the understanding of AIDS encephalopathy and the dementia that occurs in nearly one-quarter of all AIDS patients. *Journal of NeuroVirology* (2000) **6,** S90–S94.

Keywords: HIV-1; astrocytes; cell culture; latency

Introduction

Cells derived from the human fetal brain have been used for many years for studies of pathogenesis of neurotrophic viruses. For example, cultures prepared from human fetal brain were used to isolate the human polyoma virus, JCV, the etiologic agent for the demyelinating disease progressive multifocal leukoencephalopathy (PML), a significant opportunistic infection in AIDS (Stoner *et al*, 1986; Padgett *et al*, 1977). Human fetal astrocytes and astrocyte cell lines have been used to study the biology of JCV infection and help answer questions on viral pathogenesis (Major *et al*, 1985; Mandl *et al*, 1987). In HIV-1 infection of the CNS, cells of the monocyte/macrophage lineage are the predominant site for productive infection (Koenig *et al*, 1986; Gartner *et al*, 1986; Wiley *et al*, 1986). However, the mechanism(s) of neuronal loss and dysfunction leading to HIV-associated encephalopathy remain unresolved. Several studies have demonstrated the presence of infected astrocytes in pediatric (Tornatore *et al*, 1994a; Sharer *et al*, 1986) and adult brains with HIV-associated encephalopathy (Epstein *et al*, 1985; Wiley *et al*, 1986). There is evidence that astrocytes serve as reservoirs for the HIV genome (Wiley *et al*, 1986; Tornatore *et al*, 1994b; Saito *et al*, 1994) and that astrocytes may contribute to CNS pathogenesis (Atwood *et al*, 1993; Brack-Werner, 1999).

Since astrocytes are the most abundant cell type in the brain, investigation of the biology of HIV-1 infection in astrocyte cultures and the potential contribution to HIV pathogenesis in the brain seemed warranted. Astrocyte cultures derived from human fetal brain can serve as an *in vitro* model system to study their participation in viral latency and reactivation. Although cultures from fetal brain tissue contain a diverse population of cells, it is possible to prepare cultures highly enriched in astrocytes.

Phenotypic characteristics of human fetal brain cultures

Cultures derived from the human fetal brain contain multiple populations of cells including astrocytes, neurons, oligodendrocytes and microglia. In addition to these diverse cell types, there are precursor cells at different stages of differentiation. Immunocytochemical techniques using antibodies against cell type specific phenotypic markers remain the predominant method for distinguishing cells of different lineages and developmental stages. In our studies, phenotypic markers are used for cell identification: glial fibrillary acidic protein (GFAP)

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identifies astrocytes, microtubule associated proteins type 2 (MAP-2) or neurofilament proteins identify neurons, CD68 identifies microglia and galactocerebroside or myelin basic protein identifies oligodendrocytes. The techniques for separation of purified astrocytes from fetal brain have been described extensively elsewhere (Elder and Major, 1988). Human fetal brain from 8–16 weeks gestation are routinely cultured in 5% fetal bovine serum containing medium which promotes astrocyte proliferation but limits fibroblast growth. Astrocytes isolated from human fetal brain can undergo multiple cell divisions and survive approximately 20 cell generations or longer before entering a programmed cell death pathway. Cultures of human fetal brain are subjected to several cycles of rotary shaking which dislodge neurons and microglia leaving the adherent astrocytes in the culture flask. This process results in cultures which are >98% pure GFAP astrocytes which can be carried for multiple cell doublings following trypsinization. Neurons and microglia cells do not proliferate well in serum containing medium and are eliminated by the mechanical separation procedure. There are few oligodendrocytes detected in human fetal brain cultures at these early gestational ages. The culture conditions for isolation of purified astrocytes from fetal brain effectively eliminates neurons, oligodendrocytes and microglial cells.

There are different functional populations of astrocytes in the developing and adult brain based on the anatomical location and physiological properties. Subtypes of astrocytes may express different chemokine receptors, transcription factors, growth factors or cytokines. The ability to distinguish astrocyte subtypes with different functional and physiological properties may provide critical information regarding astrocyte susceptibility to HIV infection.

For example, in order to characterize human fetal brain cells in culture, we developed an antibody that specifically detects the human nestin protein,

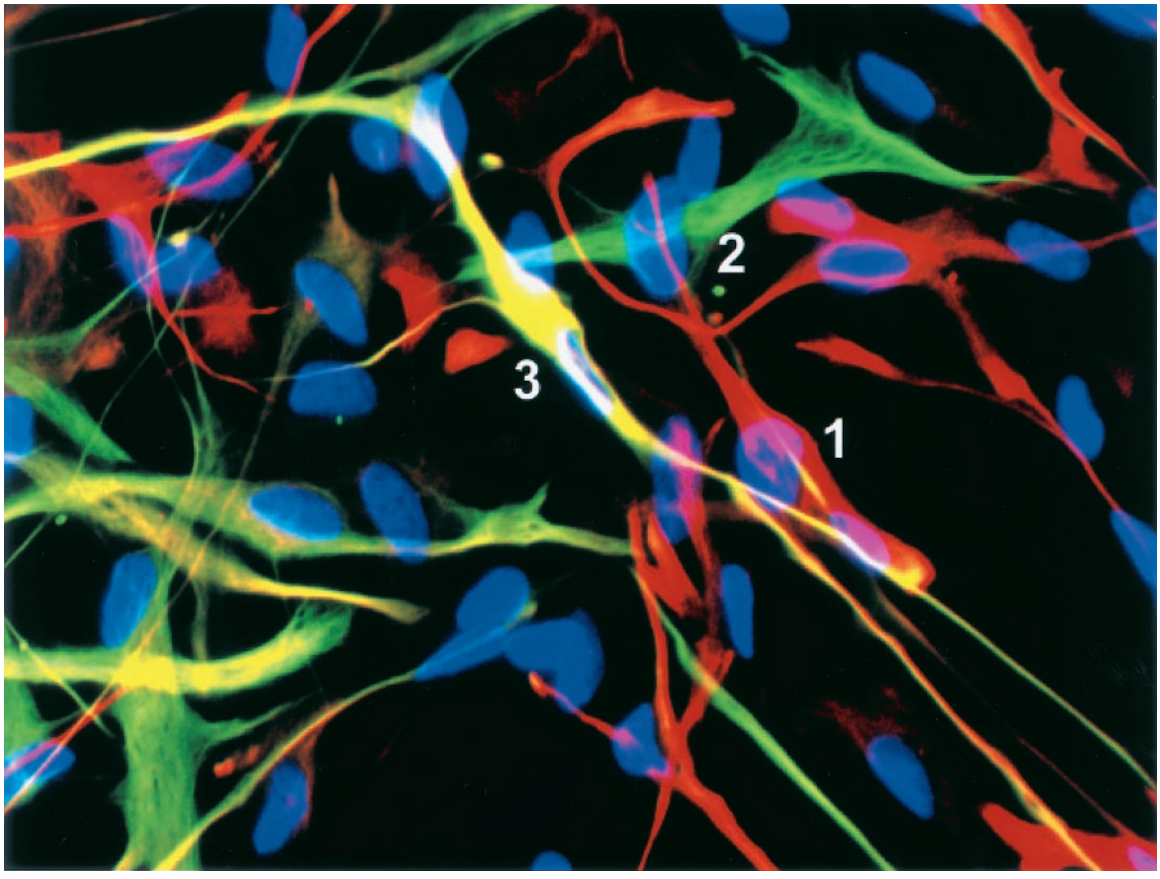

Figure 1 Double immunofluorescence staining for nestin and GFAP. Immunostaining conducted on a 10 week gestation human fetal brain cultured in serum containing medium and passaged once. Cells were immunostained using a rabbit polyclonal anti-human nestin antiserum (Messam *et al*, 2000) and TRITC-conjugated donkey anti-rabbit immunoglobulins (red). Cells were also stained using mouse monoclonal anti-human GFAP antibodies and FITC-conjugated goat anti-mouse immunoglobulins (green). Nuclei were stained with bisbenzimide (blue). The overlay of the immunofluorescence images is shown. The number 1 indicates a cell positive for nestin only and number 2 indicates a cell positive for GFAP only. The cell indicated by number 3 is co-positive for nestin and GFAP (yellow). (Magnification 300×).

an intermediate filament protein expressed in stem and progenitor cells in the CNS (Messam *et al*, 2000; Lendahl *et al*, 1990). A population of astrocytes derived from several gestational ages of human fetal brains were found to co-express nestin along with GFAP (Figure 1). As these astrocytes are further passaged and maintained in culture, they lose the expression of nestin. The use of this human specific nestin antibody in conjunction with GFAP identifies differentiation states of astrocytes in culture and has the potential of tracking neuronal differentiation as well. In the future, the development of molecular markers such as cell specific cDNAs may prove useful for the identification of cell subtypes.

Stages of HIV-1 infection in human astrocytes

The predominant cell in the brain that becomes infected with HIV-1 is the macrophage and microglia. Consequently, the M tropic strains of HIV-1 which principally utilize CD4 and CCR5 surface molecules as virus receptors, have been considered neurotropic. Infection results in multiplication of progeny virions and release of several neurotoxic molecules such as TNF-alpha, nitric oxide, arachidonic acid metabolites, and interleukins. In contrast, usually the T tropic strains of HIV-1 infect astrocytes through a CD4 independent mechanism. Some of the M tropic strains can also infect astrocytes but with less efficiency. Further, studies are being done to determine whether the XR or CCR co-receptors can be used for HIV-1 infection in astrocytes. For example, there are CXCR4 expressing astrocytes derived from human fetal brain but their susceptibility to HIV-1 does not appear more efficient than astrocytes which do not express CXCR4. Several reports have identified cell surface molecules of 260 kD and 65 kD which binds the HIV-1 gp 120 molecule (Ma *et al*, 1998; Hao and Lyman, 1999). Antibody to the 260 kD molecule blocks infection in human astrocyte cultures. It is possible that the 65 kD molecule is the monomer and the 260 kD molecule represents a tetramer complex.

The result of HIV-1 infection in astrocytes, however, is markedly different from microglia or macrophages. Using human fetal astrocytes as a model cell culture, HIV-1 infection can be described in three stages as shown in Figure 2. The initial stage of infection is productive but non-cytopathic during which proviral DNA is made and presumably integrated into chromosomes. The DNA bind-

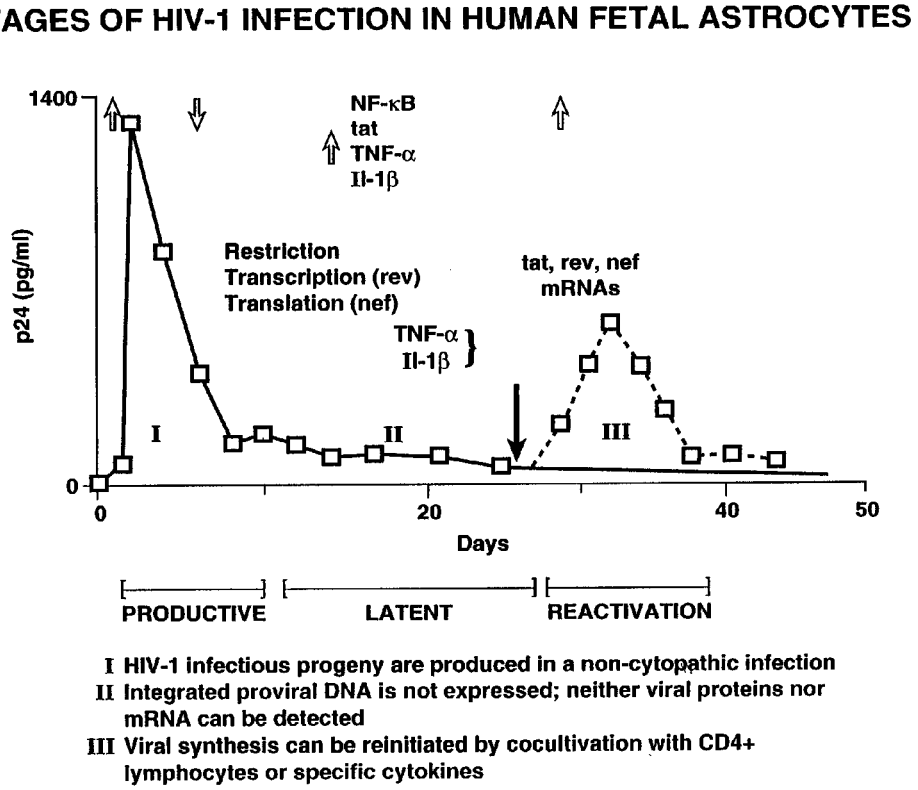

Figure 2 Graphic representation of HIV-1 infection in astrocyte cultures prepared from human fetal brain. Three stages of infection are described in relation to viral synthesis of the structural p24 protein detected by ELISA assays measured for 7 weeks from time of infection with NL4-3 strain of HIV-1. Open arrows indicate increase in DNA binding activity of the transcription factor NF-κB which occurs directly after infection or upon cell treatment with Tat, TNF-alpha or IL-1beta. Closed arrow indicates time of introduction of cytokines TNF-alpha or IL-1beta which reactivates HIV-1 mRNA synthesis and p24 production.

ing activity of the transcription factor NF-κB is greatly increased at this stage subsequent to the phosphorylation of the inhibitor I-κB complex. NF-κB then translocates from the cytoplasm to the nucleus. The HIV-1 tat protein can independently upregulate NF-κB activity which also increases the specific synthesis and release of the beta chemokine MCP-1 (Conant *et al*, 1996, 1998; Atwood *et al*, 1994). Interestingly, HIV-1 infected astrocytes demonstrate reduced glutamate uptake during this period of infection which is a vital function for neuronal survival provided by astrocytes. After several weeks, productive infection gradually diminishes with progressive loss of mRNA for structural proteins and then reduction of the mRNAs for the regulatory proteins. The HIV-1 nef mRNA is the last transcript that can be detected. This second stage results in viral latency in which neither proteins nor transcripts are made and can last for weeks to months in culture. However, at any time during the latent stage, reactivation of the viral genome can take place upon treatment with pro-inflammatory cytokines, TNF-α or Il-1β, or co-culture with CD4 positive T lymphocytes. This third stage of infection or reactivation resembles the productive phase. Progeny virions are made but again diminish with time until a latent infection is reestablished. The predominant transcripts during the reactivation stage are the tat, rev, and nef mRNAs. Quantitatively, there is less virus made after reactivation from latency compared with the initial productive infection perhaps due to loss of infected cells or loss of the integrated proviral DNA.

Reports have identified transcriptional control of the rev protein which may drive HIV-1 productive infection to a latent state (Neumann *et al*, 1995). Other studies have also identified a block in translation of transcripts for gag, rev, and nef which may result in the establishment of a latent infection (Gorry *et al*, 1999). Somewhat conflicting roles of Nef in restricting HIV-1 infection in astrocytes have been reported either upregulating HIV-1 expression (Bencheikh *et al*, 1999) or reducing the extent of infection (Gorry *et al*, 1998).

HIV-1 infection in astrocytes in brain tissue

Studies of HIV-1 infected autopsy brain tissues from pediatric encephalopathies revealed similarities to the astrocyte cell culture models (Tornatore *et al*, 1994a; Saito *et al*, 1994; Takahashi *et al*, 1996). HIV-1 infected GFAP positive astrocytes could be readily identified particularly in areas of inflammation using *in situ* PCR or DNA-RNA hybridization. Nef was the only viral protein that could be detected in a limited number of cells. However, the numbers of infected astrocytes compared with infected macrophages or microglial cells were far fewer.

Summary

Latent HIV-1 infection in astrocytes can serve as a hidden reservoir for virus in the brain. Since the inflammatory cytokines that reactivate HIV-1 in culture are also found in high concentrations in AIDS brain tissue, it is probable that virus can be released from latently infected astrocytes at any time during the long course of disease, months to years. New virus can then infect the more susceptible microglial cells or macrophages allowing a continual cycle of infection-reinfection to take place. In addition, latent infection in astrocytes reduces their neuroprotective functions while augmenting the release of monocyte chemokines. With the advent of novel HIV-1 vaccines and new drug treatments for prophylactic use, eliminating the latently infected astrocyte in the brain presents an important challenge for the selection of the appropriate viral vaccine strain and methods of drug delivery to the CNS.

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