American Journal of Pathology, Vol. 143, No. 4, October 1993

Short Communication

Productive Infection of Human Fetal Microglia by HIV-1

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Central nervous system disease is a frequent finding in both pediatric and adult AIDS. Microglia have been shown to be the major target of HIV-1 infection in the central nervous system. However, studies* in vitro *concerning susceptibility of human microglia to HIV-1 infection reported conflicting results; microglia from adult brain showed productive infection by HIV-1, whereas microglia from fetal brain did not. To investigate this further and to define the possible mechanisms responsible for this difference, we prepared highly purified human microglial cell cultures from fetuses of 16 to 24 weeks' gestation and exposed them to monocytotropic (HIV-1$_{JR-FL}$ and HIV-1$_{JR-CSF}$) isolates of HIV-1. Culture supernatants were examined for the presence of p24 antigen for a 4-week period after viral exposure. Concurrently, potential cytopathic effects and cellular viral antigen expression (gp41 and p24) were examined by light microscopy in combination with immunocytochemistry. The results showed that human fetal microglia can be productively infected by HIV-1 as judged by p24 antigen capture assay, syncytia formation, and gp41 and p24 immunoreactivity of infected microglia. In addition, by electron microscopy, numerous viral particles characteristic of HIV-1 were present both in the intracellular and extracellular compartments. Uninfected cultures or astrocytes overgrown in the microglia cultures did not show evidence of infection under identical experimental conditions. These data demonstrate that human fetal microglia, like their adult counterparts, are susceptible to HIV-1 infection* in vitro *and can support the production of virus. (Am J Pathol 1993, 143:1032–1039)

A large percentage of adult and pediatric AIDS patients exhibits signs and symptoms of nervous system disease.[1] Human immunodeficiency virus-1 (HIV-1), the causative agent of AIDS, is believed to be neurotropic, but issues related to its neurotransmission, neural cell infectivity, and neurovirulence remain controversial. Within the central nervous system (CNS), HIV-1 has been localized almost exclusively to multinucleated giant cells and microglia.[2–4] There appears to be, however, a disparity in the frequency of HIV-1 protein or nucleic acid expression among brains of different ages: virus has infrequently been detected in the brains of young (<2 years old) AIDS patients or in fetuses of HIV-seropositive women.[4–6] Moreover, a recent study reported a failure of infection of primary cultures of human embryonic microglia by HIV-1,[7] whereas primary cultures to adult human microglia have been shown to be productively infected by HIV-1.[8] We were intrigued by the possibility that fetal microglia may indeed have a different susceptibility for HIV-1 infection or ability to support a productive infection, compared with microglia from adult CNS. We wished to confirm this possibility using human fetal microglia cultures established at this laboratory.[9] The results reported here show that human fetal microglia have an essentially similar susceptibility to HIV-1 infection as do their adult counterparts.

Supported by NIMH grants MH 47667 and MH 46815.

Accepted for publication June 11, 1993.

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Materials and Methods

Tissue

Human fetal CNS tissue was obtained as part of an ongoing research protocol that has been approved by the Albert Einstein College of Medicine Committee on Clinical Investigations and the City of New York Health and Hospitals Corporation. Informed consent was obtained from all participants. Fetal brains were obtained after elective terminations of pregnancy from normal women with no risk factors for HIV-1 infection. Fetal ages were determined by measurement of foot length.

Cell Cultures

Highly enriched microglia cultures were prepared using second trimester human fetal brains, as described previously.[9] Briefly, brain tissue was minced and digested in Hanks' balanced salt solution containing 0.05% trypsin and DNAse for 45 minutes at 37 C, and the digest was passed sequentially through 230- and 130-μ nylon meshes. Cells were washed in serum-containing medium and seeded at 4×10^7 cells/75 cm^2 tissue culture flask in Dulbecco's modified Eagle's medium (with 4.5 g/L glucose, 584 mg/L L-glutamine, and 25 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, Whittaker, Walkersville, MD) containing 10% heat-inactivated fetal calf serum, in a 95% air/5% CO_2 humidified incubator. After 2 weeks *in vitro*, floating microglia were collected and reseeded at 0.2×10^6 cells/well in 24-well plates. All microglia cultures were washed with fresh media after 1 hour to remove nonadherent cells. Using CD68 and glial fibrillary acidic protein (GFAP) as cell type specific markers, microglia cultures contained less than 1% astrocytes. The microglia culture media consisted of Dulbecco's modified Eagle's medium/10% fetal calf serum, supplemented with 2 U/ml granulocyte-macrophage colony-stimulating factor (Genzyme, Cambridge, MA).

HIV-1 Infection

Microglial cultures (14 to 20 days *in vitro*) were exposed for 16 hours at 37 C to 0.5 ml of cryopreserved (−80 C) HIV-1 isolates ($HIV\text{-}1_{JR\text{-}FL}$[10] or $HIV\text{-}1_{JR\text{-}CSF}$) previously propagated in primary human fetal liver macrophages, at a multiplicity of infection of 0.01 (2000 infectious units/200,000 microglia). Nonabsorbed virus was removed by extensive washing of the culture with Hanks' balanced salt solution.

P24 Antigen Capture Enzyme-Linked Immunosorbent Assay

P24 *gag* protein concentration in the tissue culture supernatants was determined using the commercial enzyme-linked immunosorbent assay kit supplied by DuPont (Wilmington, DE).

Immunocytochemistry

Detection of HIV-1-specific proteins was achieved using monoclonal antibodies to gp41 (Genetic Systems, Seattle, WA) or p24 (DuPont). Microglia or astrocytes were identified by monoclonal antibodies to CD68 (EBM-11: DAKO, Carpinteria, CA) or GFAP (Boehringer-Mannheim, Indianapolis, IN), as previously described.[9] Cells grown in 24-well plates were fixed in ice-cold methanol for 30 minutes, washed in phosphate-buffered saline, and preincubated in 10% normal goat serum/10% normal human serum/phosphate-buffered saline for 1 hour to block nonspecific antibody binding. Primary antibodies were diluted in the same buffer at 1:800 (gp41), 1:300 (p24), 1:50 (EBM-11), and 1:100 (GFAP) (all mouse IgG1), and applied for 16 hours at 4 C. Cells were then washed and incubated with peroxidase conjugated goat anti-mouse IgG1 (Fisher Scientific, Pittsburgh, PA) at 1:250. The immunolabeled cells were visualized using diaminobenzidine as chromogen. For double-labeling immunofluorescence studies, rabbit IgG to GFAP (Biocell) at 1:1000 or HAM-56 (marker for human macrophages: mouse IgM, Enzo Diagnostics, New York, NY) at 1:100 was added simultaneously with anti-p24 for 30 minutes at 4 C, followed by isotype-matched fluorescein isothiocyanate- or rhodamine-conjugated goat antibodies for another 30 minutes.

Cell Counting

To assess cell death in HIV-1 infected microglial cultures, cultures were immunostained for CD68 at 30 days after exposure to HIV-1, and the total number of nuclei in CD68 (+) cells per microscopic field was compared with that in control cultures. Since in infected cultures many microglial cells showed cell fusion (syncytia), we chose nuclear counting rather than cell counting as a correlate of cell survival. In separate experiments, cultures at varying time points after HIV-1 exposure were stained with 0.4% typan blue for 5 minutes, washed, and then immunolabeled for CD68 as described above, to visualize dead microglia in culture. Trypan blue (+)/

CD68(+) cells were counted per microscopic field in triplicate wells, and the numbers in infected wells were compared with those in control wells.

Electron Microscopy

Microglia seeded at 0.2 × 10^6 cells/ well into 4-well plastic chamber slides (Nunc, Naperville, IL) were exposed to HIV-1_{JR-FL} or HIV-1_{JR-CSF}, as described above. At 20 days after inoculation, the monolayers were fixed in Trump's solution (1% glutaraldehyde/4% paraformaldehyde in phosphate-buffered saline) for 24 hours at 4C, post-fixed in 1% osmium tetroxide, dehydrated in ascending ethanol, and embedded as a monolayer in Epon-araldite. Ultrathin sections were counterstained with lead citrate and uranyl acetate, and examined with a Siemens 102 electron microscope.

Results

Phase Contrast Microscopy

Detailed morphological and immunocytochemical characterization of human fetal microglia (control cultures) has been reported previously.[9] At 5 to 7 days after-inoculation with monocytotropic isolates of HIV-1 (HIV-1_{JR-FL} and HIV-1_{JR-CSF}), the microglia cultures began to show syncytia formation (average, 10–20 nuclei/cell), a typical cytopathic effect of HIV-1 infection (Figure 1). The multinucleated giant cells and mononuclear microglial cells formed clusters reminiscent of microglial nodules in the brains of patients with AIDS.[2,4] These changes were progressive and peaked at 2 to 3 weeks after inoculation, correlating with peaks in p24 levels (see below). In contrast, microglia in control cultures maintained uniform, small perikarya and were often ramified in foci of astrocyte overgrowth (Figure 1).

Due to their high mitotic index, the small number of astrocytes that contaminate microglial cultures can become the major cell type over a 3- to 4-week period.[9] Under current experimental conditions, expression of HIV p24 immunoreactivity and/or syncytia formation was limited to microglial cells, as studied by double-labeling immunofluorescence; GFAP/p24 double-positive cells were not detected, consistent with the findings by others that productive HIV-1 infection in primary brain cell cultures is limited to macrophage/microglia.[8]

Some cell death and detachment was detected in both infected and uninfected cultures over time; however, we did not see massive cell death, as described in HIV-1-infected T cells or adult microglia.[8]

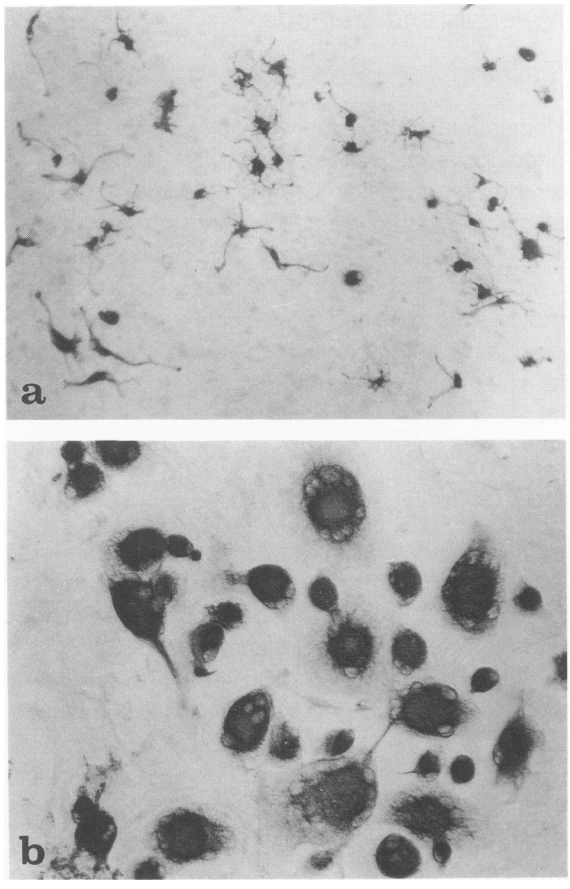

Figure 1. *Human fetal microglial culture at 30 days after inoculation with a monocytotropic strain HIV-1_{JR-FL}. Control uninfected* (a) *and infected* (b) *cultures were immunostained for EBM-11, a marker for microglia, demonstrating small ramified microglia in* (a), *and numerous multinucleated giant cells in HIV-1-exposed culture* (b). *Note peripherally arranged, unstained nuclei in* (b). *Photograph was taken from areas of astrocyte growth (background monolayer of unstained cells)* (a, ×150; b, ×300).

In fact, nuclear counting (since there was pronounced multinucleation by cell fusion in HIV-1 infected microglial cultures, nuclear counting rather than cell counting was considered a proper method of evaluating cell loss) revealed that there was essentially no difference in microglial nuclear number between infected and uninfected cultures examined at 30 days after inoculation (70 ± 16 versus 77 ± 12 per ×300 microscopic field: mean ± SD). In addition, trypan blue exclusion (see Materials and Methods) performed at 14 and 21 days after inoculation revealed no difference in the number of trypan blue (+) microglia between infected and control cultures (0–1 per × 300 microscopic field in both). Moreover, by ultrastructural criteria (see below), as well as light microscopic appearance, no definite evidence for cellular degeneration such as vesiculation of membranous organelles or clumping of nuclear chromatin was seen in the infected cultures.

Immunocytochemistry

Identity of microglia in cultures was confirmed by CD68 or HAM-56 immunocytochemistry; it showed that the majority of microglia in infected cultures had formed multinucleated giant cells by the end of 3- to 4-week culture period (Figure 1). A subpopulation (~20–30%) of microglia in infected cultures exhibited immunoreactivity for HIV-1 p24 or gp41, at 3- to 4-weeks after inoculation (Figure 2). p24 immunoreactivity was sometimes expressed as punctate perinuclear staining.

Electron Microscopy

HIV virions with characteristic eccentric cone-shaped cores, ranging from 85 to 150 nm in diameter, were numerous in the multinucleated microglial cells, most of them in intracellular membrane bound compartments, as previously described in monocytes[11] (Figure 3). Immature virions and budding forms were also present. Extracellular virions were present, but rare compared with intracellular ones. In addition, the microglial cells contained numerous lipid droplets, primary lysosomes, tubular mitochondria, rough endoplasmic reticulum, filaments, and ribosomes, similar to uninfected microglia.[9] Curved electron-dense profiles resembling confronting cylindrical cisterns,[12,13] probably a derivative of endoplasmic reticulum, were numerous in some cells. Occasionally, zones of organelle-free, complex membrane infoldings were seen between two adjacent cells; at times, viral particles were present between adjacent infolded plasmalemma.

P24 Antigen Capture Assay

Culture supernatants were collected from triplicate wells, once or twice per week, with complete replacement by fresh media (1 ml/well). Typically, p24 levels in cultures exposed to monocytotropic stains of HIV-1 rose at 7 to 10 days, peaked around 2 to 3 weeks, and then fell (Figure 4).

Discussion

In this report, we present evidence for productive HIV-1 infection of human fetal microglia in culture.

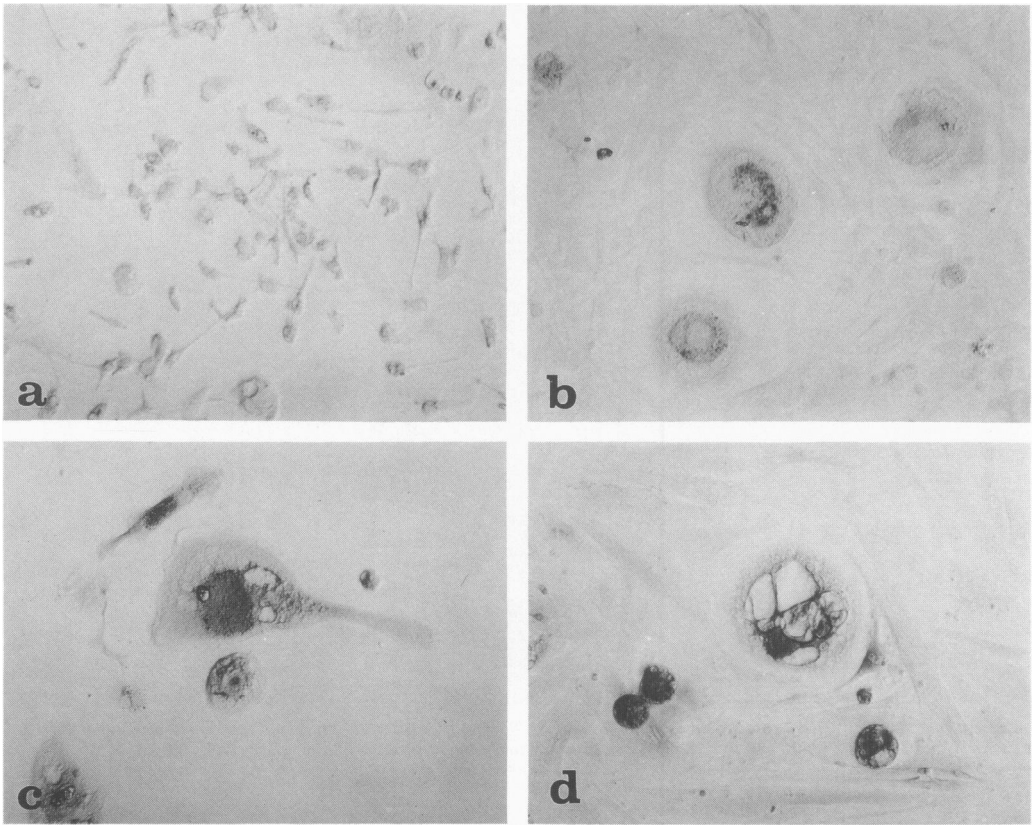

Figure 2. *Human fetal microglia cultures as in Figure 1 were immunostained for HIV-1 gag protein p24* (**a–c**) *and envelope protein gp 41* (**d**). *Control uninfected culture* (**a**) *shows microglial cells devoid of p24 immunoreactivity, whereas infected cultures* (**b–d**) *show multinucleated cells with varying degrees of HIV protein expression, often localized in areas adjacent to nuclei. Astrocytes remain unstained* (**b** *and* **d**). (**a**, ×225; **b**, ×300; **c**, ×225; **d**, ×225).

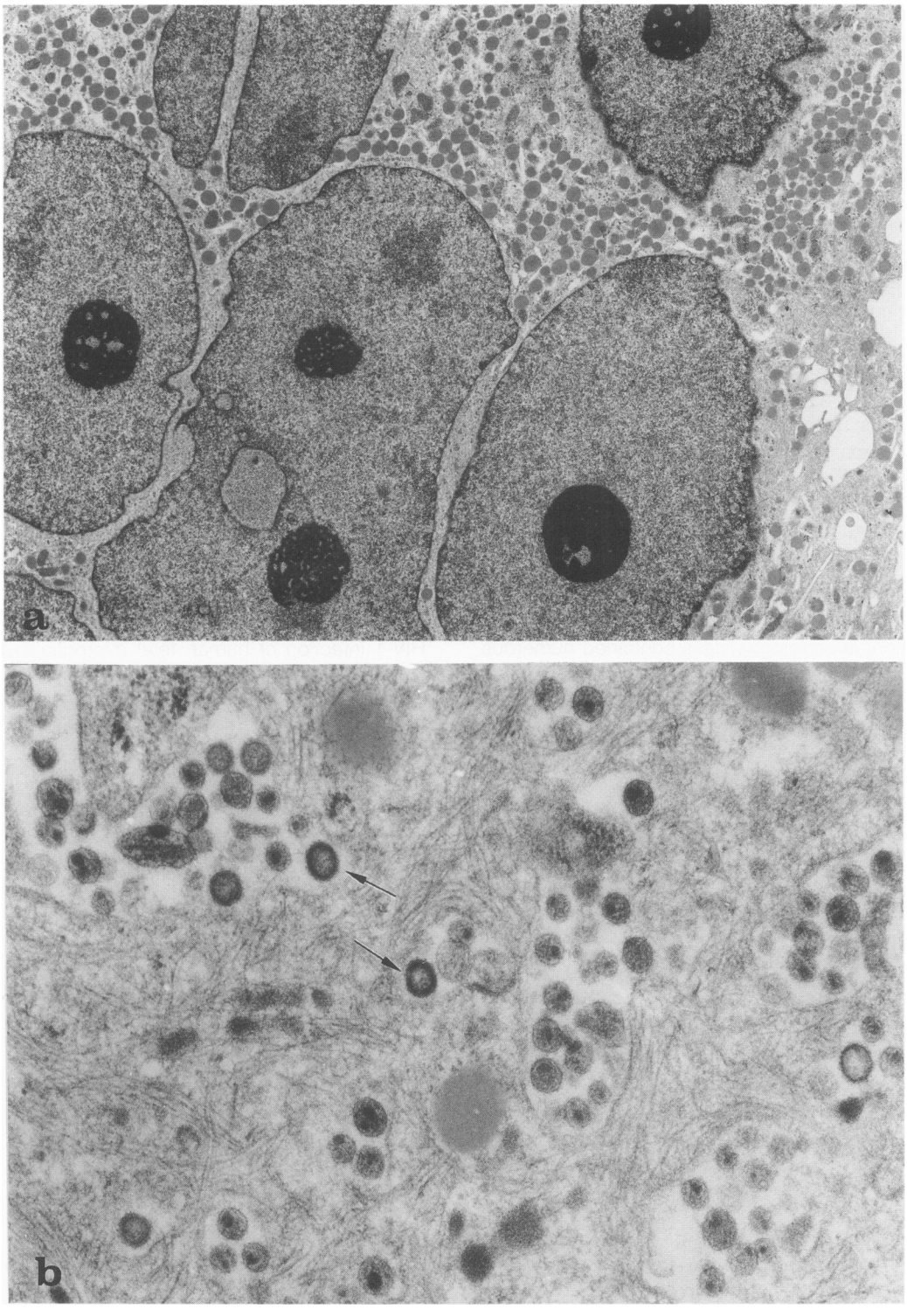

Figure 3. *Electron microscopy of HIV-1-infected microglial culture at 20 days after inoculation. A multinucleated microglial cell* (**a**) *containing numerous mature, and some immature* (**arrows**) *viral particles in intracellular membrane-bound spaces* (**b**). *The virions show pleomorphic, eccentric, cone-shaped cores, characteristic of HIV* (**a**, ×4,500; **b**, ×60,000).

Typical cytopathic changes were associated with production of mature virions, as well as release of abundant viral proteins into the culture supernatant. The time course of infection was also similar to that reported in cultured peripheral monocytes[14] and adult brain microglia[8] infected with monocytotropic

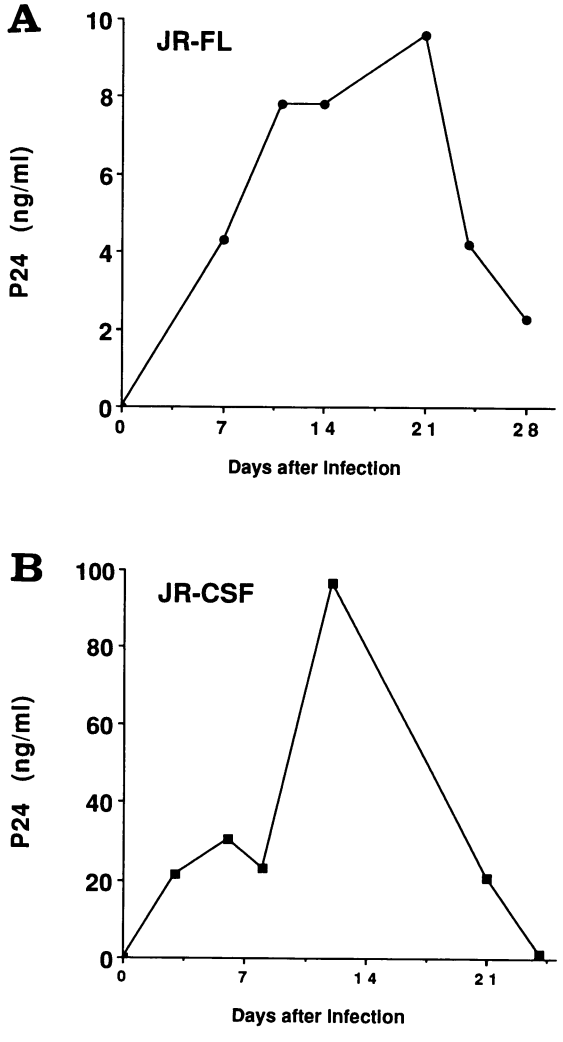

Figure 4. *p24 gag antigen capture assay in human fetal microglial culture. Highly enriched microglial cultures seeded at 2 × 10⁵ cells/well in 24-well plates were exposed to 2000 infectious units (MOI 0.01) of HIV-1$_{JR\text{-}FL}$* (A) *or HIV-1$_{JR\text{-}CSF}$* (B) *isolates for 16 hours, as described in Materials and Methods. Supernatants were collected at indicated time points from uninfected and infected cultures, and p24 levels were measured using a commercial enzyme-linked immunosorbent assay kit as described. Results are representative of two separate experiments (two with HIV-1$_{JR\text{-}FL}$, two with HIV-1$_{JR\text{-}CSF}$). No p24 antigen was detected in control, uninfected cultures (not shown).*

strains of HIV-1. By electron microscopy, numerous viral particles characteristic of HIV-1 were present both in intracellular and extracellular compartments. Unlike T cells infected with HIV-1 *in vitro*, where viral assembly is almost exclusively associated with the cell surface, monocytes infected with HIV show predominant localization of progeny virus within the cytoplasm.[11] These findings were thought to correlate with lytic infection of T cells, and chronic non-lytic infection of monocytes suggesting monocytes as a viral reservoir.[11,14] In the current HIV-infected human fetal microglial cultures, viral particles were predominantly located intracellularly, and evidence for cell death was minimal. This is similar to the findings in the brains of AIDS patients, where multinucleated giant cells sometimes harbor large numbers of intracellularly located viruses (refs. 3 and 11 and unpublished observations). Decline in p24 levels at the later phase of infection in fetal microglial cultures (Figure 4) may also suggest a beginning of latent or persistent low-level infection, as has been shown in HIV-1-infected monocytes.[11,14] Survival of HIV-1 infected microglia in fetal cultures contrasts with the reported progressive cell death in HIV-1 infected adult microglial cultures.[8] This may have been due to the differences in culture technique, such as initial cell plating density and/or cell number (fetal cultures contained ~100 times more cells/well) or may have been due to the presence of coexisting astrocytes in long-term fetal microglial cultures, which may have supported the survival of microglia via production of macrophage-colony stimulating factor.[15]

Although a previous report by Peudenier et al[7] proposed differential susceptibility of peripheral monocytes and microglia to HIV-1 infection and concluded that cultured embryonic microglia are resistant to HIV-1 infection, the present study does not support this conclusion. The reasons for the discrepancy are not clear, but factors such as maturity of the host tissue (first versus second trimester fetus), different infectivity among various HIV-1 isolates, or differences in viral titers used in experiments may be responsible. For instance, a recently published study indicates that cytokine production by first trimester human embryonic glial cells[16] is quite different from that by second trimester glial cells.[17] Whereas tumor necrosis factor-α messenger RNA and protein were shown to be produced by second trimester microglia and astrocytes after proper stimulation, neither cell type cultured from early embryonic tissue showed evidence for production of tumor necrosis factor-α. Cytokines such as tumor necrosis factor-α or interleukin-1β have been shown to induce expression of HIV-1 in latently infected cell lines and primary cells[18–20]; therefore, the differences in the ability to produce certain cytokines may have affected the cell's ability to maintain productive infection by HIV-1. The possibility also exists that levels of CD4, a cellular receptor for HIV-1, on microglia may vary during maturation. Preliminary studies suggest that microglia used in this study express little or no detectable CD4 antigen when examined by immunofluorescence and FACS analysis; further investigations are under way using more sensitive methods to detect

CD4 expression by these cells. The current study also suggests that differential expression of HIV-1 among adult, pediatric, and fetal CNS in AIDS may be explained by factors other than target cell susceptibility. Other elements, such as maturity of the immune system and/or CNS microenvironment, may have a bearing on the sequence of events that ultimately leads to productive infection and HIV encephalitis.

In summary, we have shown *in vitro* infection of primary cultures of fetal microglia by HIV-1. This is further proof that microglial cells in the CNS have specific susceptibility for HIV-1, and may be the key cell type in the pathogenesis of the CNS dysfunction in AIDS. Free viruses or HIV-infected peripheral monocytes entering the CNS can directly infect intrinsic microglia of the CNS; infected microglia can serve as viral reservoir, further propagating the virus within the CNS. Infected and uninfected microglia can also secondarily affect the CNS tissue via amplification of cytokine cascades[21,22] or the production of potentially neurotoxic substances.[23–25] The human microglial culture system may serve as a model to study further mechanisms of HIV infection and pathogenesis of CNS dysfunction.

Acknowledgments

We thank Ms. Mila Losev for excellent technical assistance.

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