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Analysis of human endothelial cells and cortical neurons for susceptibility to HIV-1 infection and co-receptor expression

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Neuronal cell death is believed to be the underlying cause of neurological diseases and AIDS dementia often seen in human immunodeficiency virus (HIV) infected patients. The means by which HIV invades the brain is still unknown and the mechanism of neuronal cell death remains to be elucidated. The aim of this study was to determine if direct infection of human brain endothelial cells and neurons play a role in viral invasion of the brain and neuronal cell death, respectively. To this effect, we evaluated human brain microvascular endothelial cells (HBMEC) and human cortical neurons (HCN) for the expression of HIV co-receptors and their susceptibility to HIV-1 infection. While both HBMEC and HCN failed to express any CXCR4 and CCR5 on their cell surface, as assessed by flow cytometry, RT–PCR revealed the presence of CXCR4 and CCR5 mRNA in HBMEC but not in HCN. Two dual tropic HIV-1 primary isolates failed to infect both cell types as determined by p24 antigen capture ELISA, RT–PCR and DNA PCR. These data support the hypothesis that no productive infection of HBMEC and HCN occurs *in vitro* and suggest that other cell types are the primary focus of HIV-1 infection in the brain. *Journal of NeuroVirology* (2000) **6,** 519–528.

Keywords: HIV infection; neurons; brain endothelial cells; HIV co-receptor

Introduction

The brain represents a potential target in HIV-1 infection (Price *et al*, 1988). For patients in the advanced stage of infection, CNS dysfunction is a common and important cause of morbidity, and leads to progressive dementia, cerebral atrophy and death. HIV-1 associated dementia complex is characterized by cognitive, motor and behavioral dysfunction (Navia *et al*, 1986; MacArthur, 1987; Brew, 1993). Although opportunistic infections, when present, may contribute to this neurologic syndrome, evidence suggests that HIV-1 is the primary etiologic agent in the pathogenesis of HIV-1 associated dementia complex. Furthermore, studies have confirmed the presence of HIV in various parts of the brain and the central nervous system (Wiley *et al*, 1986; Sinclair *et al*, 1994; Takahashi *et al*, 1996; An and Scaravilli, 1997). The underlying cause of AIDS dementia appears to result from a significant loss of neuronal cells during infection and subsequent disease progression (Giangaspero *et al*, 1989; Ketzler *et al*, 1990; Gray *et al*, 1991). Two issues that are central to understanding the pathogenesis of AIDS dementia are how HIV-1 enters the brain, and by what mechanism(s) HIV-1 induces neuronal cell death.

A number of investigations have described the toxic effects of certain cytokines and HIV encoded proteins, such as gp120 on tat on neurons (Brenneman *et al*, 1994; Meucci and Miller, 1996; Aggoun-Zouaoui *et al*, 1996; Kanmogne *et al*, 1999). It has been postulated that these toxic substances resulting from HIV infection are involved directly in neuronal cell death. Another potential mechanism of neuronal cell death may be the secretion of

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neurotoxic products by HIV infected macrophages and microglia cells in the brain (Wesseling *et al*, 1993; Pulliam *et al*, 1994). Recent reports have described the expression of the HIV co-receptors on some neuronal cell populations (Rottma *et al*, 1997; Hesselgesser *et al*, 1997; Lavi *et al*, 1997; Sanders *et al*, 1998; Vallat *et al*, 1998; Klein *et al*, 1999), however, evidence that HIV can directly infect neurons, either *in vitro* or *in vivo* is not convincing. The exact mechanism(s) of neuronal cell death in AIDS dementia remains to be determined.

Whether HIV-1 can infect brain endothelial cells and/or damage the blood brain barrier is controversial. Efficient infection of brain microvascular endothelial cells by a neuroinvasive SIV strain was recently reported (Strelow *et al*, 1998). It has also been suggested that human brain endothelial cells can support HIV-1 replication and thus, could act as a reservoir for viral propagation and dissemination in the brain (Moses *et al*, 1993; Moses and Nelson, 1994; Poland *et al*, 1995; Gilles *et al*, 1995). However, other investigations have reported no evidence of brain endothelial cell infection by HIV-1 (Nottet *et al*, 1996). Thus, the mechanism of HIV entry into the brain and the role of brain endothelium in HIV invasion of the brain remain to be elucidated.

Several members of the seven-transmembrane domain family of chemokine receptors can function as co-receptors for HIV infection (Deng *et al*, 1996; Dragic *et al*, 1996). In addition to the CD4 receptor, the CCR5 co-receptor is utilized by most non-syncytium inducing macrophage-tropic strains, while CXCR4 is used by most syncytium inducing T lymphocyte-tropic strains. The relationship between co-receptor usage and HIV-1 phenotype does not appear to be strict. Certain HIV-1 strains have been identified that can utilize CCR5 and CCR3 in cell lines transfected with these genes, but cannot infect primary macrophages. Other members of the chemokine receptor family, CCR3 and CCR2b have also been shown to act as co-receptors for a limited number of primary HIV-1 isolates. Some HIV-1 isolates are dual tropic and can use both CCR5 and CXCR4 co-receptors (Simmons *et al*, 1996). The presence of these co-receptor molecules on brain endothelial and neuronal cells could facilitate entry of HIV into these cells and produce infection.

The goal of this study was to evaluate human brain microvascular endothelial cells (HBMEC) and human cortical neurons (HCN-1A) for the expression of the HIV-1 co-receptor molecules and determine whether these cells could be infected with primary isolates of HIV-1. The data presented herein demonstrated that HBMEC failed to express CXCR4 and CCR5 on their surface by flow cytometry. The HGN also failed to express CCR5 and CXCR4 on their surface. By reverse transcriptase (RT)–PCR, CXCR4 and CCR5 mRNA was detected in HBMEC but not in HCN. Two dual tropic primary HIV-1 isolates from different clades failed to infect either cell type *in vitro*. These studies support the notion that these two cell types present in the human brain can not support productive HIV-1 infection *in vitro*.

Results

Detection of CXCR4 and CCR5 mRNA in HBMEC but not HCN

To determine whether CXCR4 and CCR5 mRNA was present in brain endothelial cells and neurons, RT–PCR was performed. Amplification products were run on a 2% agarose gel and stained with ethidium bromide. Results of the representative reactions using CXCR4- and CCR5-specific primers are shown in Figure 1A and B, respectively. CXCR4 RT–PCR generated amplicons of the expected size in endothelial cells, but not in neurons. Controls for CXCR4 included HOS cell line expressing CXCR4 (lane 2) and human PBMCs (lane 3). RT–PCR targeting CCR5 was negative for both brain endothelial cells and neurons (Figure 1B). However, when endothelial cells were treated with 10 ng/ml TNF-α for 24 h prior to RNA extraction, CCR5 RT–PCR generated an amplicon of the expected size (Figure 1C). Brain endothelial cells had less CCR5 RNA copies compared to CXCR4, as assessed by the intensity of the amplified bands. These experiments were repeated five times and results were consistent. After each CCR5 or CXCR4 RT–PCR, equal amounts of cDNA (5 μl) were used in a PCR reaction with primers specific for the β-actin gene. Results were positive for each sample, and the intensity of the amplicons showed that equal amounts of RNA and cDNA were used for reverse transcription and PCR respectively (Figure 1D).

Lack of expression of HIV-1 co-receptors on the surface of human brain endothelial cells and neurons

The ability of HIV-1 to infect human endothelial cells and neurons depends on the cells' ability to express viral receptors/co-receptors on their surface. Therefore, we evaluated the expression of CCR5 and CXCR4 on brain endothelial cells (HBMEC) and neurons by flow cytometry. HOS cell lines expressing CCR5 and CXCR4 were used as positive controls. Representative flow cytometry experiments are shown in Figure 2. No detectable CXCR4 (A and B) or CCR5 (C) was expressed on either endothelial cells or neurons. HBMEC had similar relative fluorescence units as the negative control cells (HOS.pBABE-puro), with only 8% of HBMEC staining positive for CXCR4. This compared to 44% in HOS.cells expressing CXCR4. Some experiments showed expression of CXCR4 on neurons (not shown), but results were not consistent as repeated experiments with different cell lots

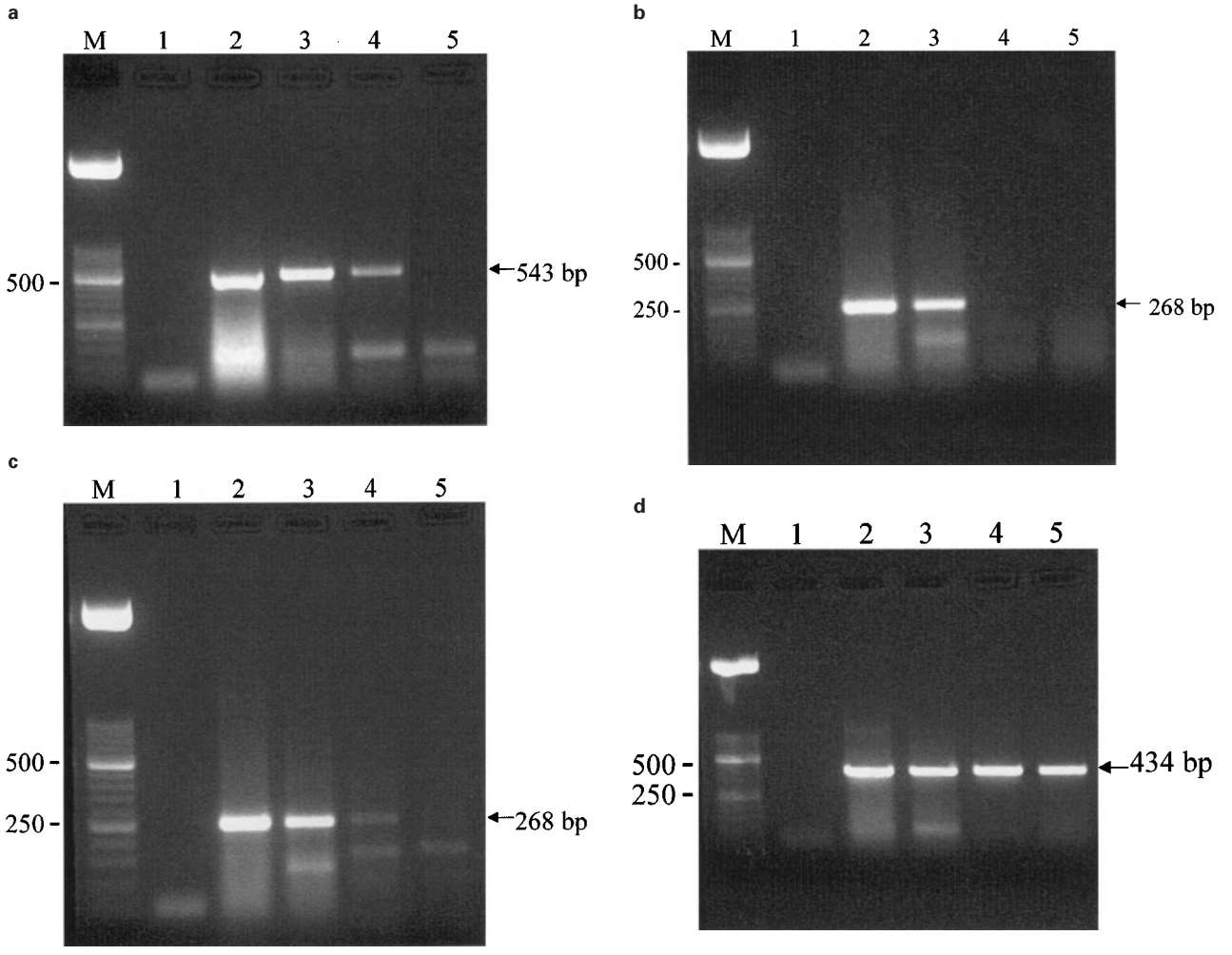

Figure 1 Expression of CXCR4 (**A**) and CCR5 (**B**) mRNA in HBMEC and HCN cells. Final RT–PCR products were run on a 2% agarose gel stained with ethidium bromide. Lanes 1–5 are respectively: negative control, positive control [HOS.CXCR4 in A and HOS.CCR5 in **B**, **C** and **D**], human PBMC, HBMEC and HCN. M is a 50 bp ladder (Boehringer Mannheim). (**C**) CCR5 RT–PCR, using RNA from HBMEC treated with 10 ng/ml TNF-α, for 24 h. (**D**) Control PCR, using β-actin primers.

or cells at different passage often gave negative results, with similar fluorescence intensity as unstained neurons or cell stained with an irrelevant antibody (Figure 2B).

Inability of HIV-1 to infect brain endothelial cells and neurons

To determine if human brain endothelial cells and neurons could be infected by HIV-1 *in vitro*, we carried out infectivity experiments. Cells were grown to 80% confluency, then infected with two different primary HIV-1 isolates (Clades B and D), and culture supernatant collected every 3 or 4 days as described in Material and methods. The result of the p24 antigen capture ELISA in HIV-infected endothelial cells is shown in Figure 3. The p24 antigen level was above 20 pg/ml on day 3 post-infection (p.i.), but dropped and constantly remained below 4 pg/ml from day 6 p.i. To rule out the fact that any positive result observed might be due to residual infection from the viral inoculum, some cells were sub-cultured by trypsinization at day 3 p.i. This did not have any impact on p24 antigen levels, because results were similar for non-trypsinized cells and cells sub-cultured by trypsinization after HIV infection. Four different experiments gave similar results: the range of the p24 antigen was between 22–45 pg/ml at day 3 p.i., and between 6–0 pg/ml from day 6 to day 18 p.i. HIV-1 infection of neurons gave similar results, with p24 antigen dropping from 18 pg/ml on day 6 p.i. to undetectable levels on day 12 p.i. (data not shown).

To confirm our p24 antigen results, we evaluated culture supernatant from infected cells for the presence of RT activity. No sample from infected endothelial cells and neurons showed any detectable RT activity (Figure 4). In other infectivity experiments, brain endothelial cells were treated with 10 ng/ml TNF-α for 24 h, and then infected as above. Culture supernatant was collected every 3

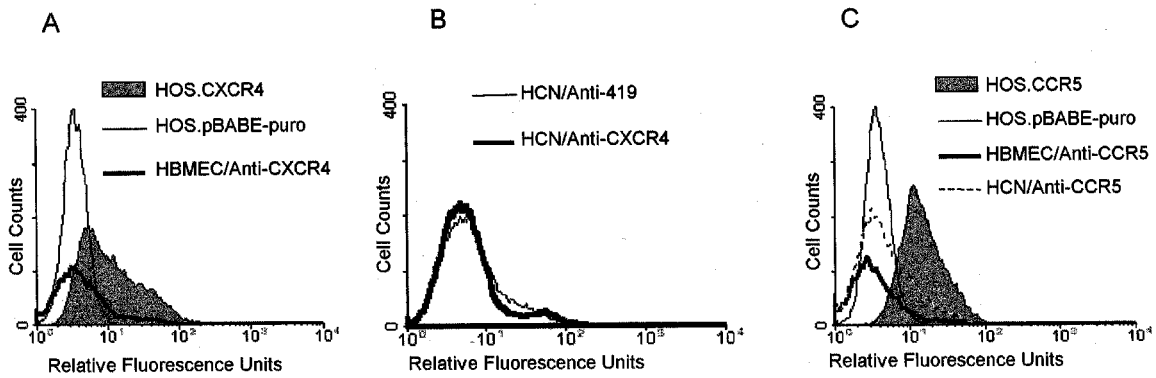

Figure 2 Lack of surface expression of CCR5 and CXCR4 chemokine receptors on human endothelial cells and neurons. Confluent cells were detached and stained with monoclonal antibody specific for CXCR4 (**A** and **B**), CCR5 (**C**), an irrelevant monoclonal antibody specific for SV-40 large tumor antigen (**B**) and analyzed by flow cytometry. HOS cells lines expressing CXCR4 and CCR5 were used as positive control, and HOS.pBABE-puro cell line as negative control.

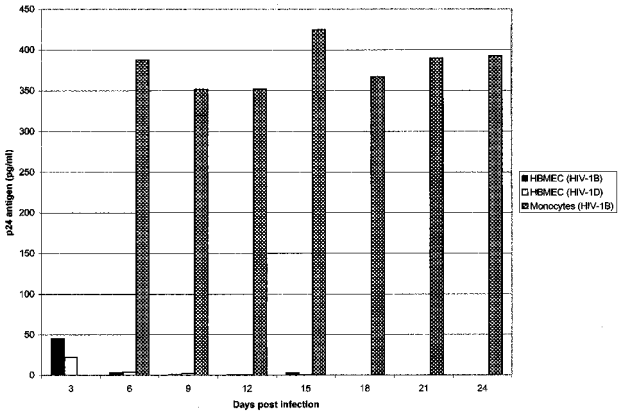

Figure 3 Lack of HIV-1 replication in HBMEC. Cells were infected with two primary HIV-1 isolates as described. Culture supernatant was collected at regular intervals and tested for p24 antigen. The p24 antigen remained below 4 pg/ml after day 3 p.i. On the contrary, monocyte/macrophages infected with the same HIV isolates constantly had p24 antigen levels above 350 pg/ml from day 6 to day 24 p.i.

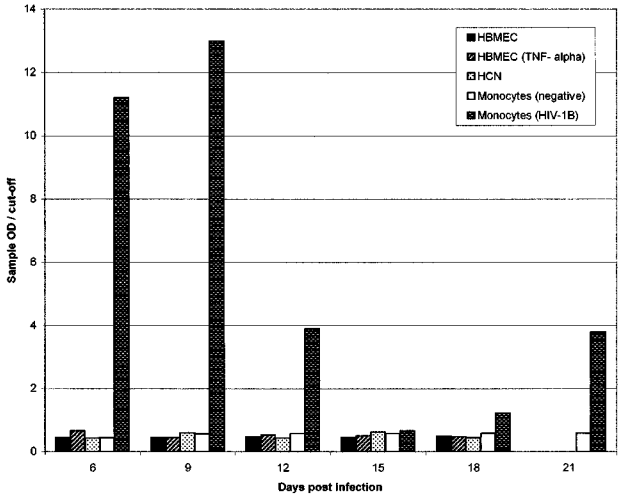

Figure 4 Reverse transcriptase activity in brain endothelial cells and neurons infected with HIV-1, clade B. Culture supernatant was collected every 3 days from infected cells and reverse transcriptase activity measured as described in Materials and methods. The cut-off=mean negative control (donor plasma)+4 s.d. s.d. is the standard deviation of the OD values of 80 HIV seronegative donors. Non-infected monocyte/macrophages were used as negative control, and HIV-1 infected monocyte/macrophages as positive control. Both HBMEC and HCN had no detectable reverse transcriptase activity from day 6 to day 18 p.i.

days up to day 21, and tested. All samples were negative for p24 antigen and RT activity (Figure 4). When tested by RT–PCR, HIV-1 RNA was detected in culture supernatant at 3–5 days post infection (Figure 5A). Samples collected after day 5 were generally negative (Figure 5B). All samples from cells that had been sub-cultured after HIV infection were also negative by RT–PCR (data not shown). At 15 or 17 days post-infection, all cells were harvested, and DNA extracted and analyzed by PCR using HIV-1 gag specific primers. All samples were repetitively negative (data not shown). Together, these data suggest that the observed p24 antigen and positive RT–PCR at early time points post infection, was the result of residual virus from the viral inoculum.

Discussion

The mechanisms whereby HIV-1 invades the brain and causes dementia remain to be defined. Central to defining these events is understanding the effect of HIV-1 on brain endothelial cells and neurons.

The ability of HIV-1 to infect vascular endothelium is controversial. Endothelial cells from saphenous vein and aorta are not permissive for HIV-1 infection (Lafon *et al*, 1992; Ades *et al*, 1993), while microvascular endothelial cells from the liver and kidney are infectible via CD4-dependent and

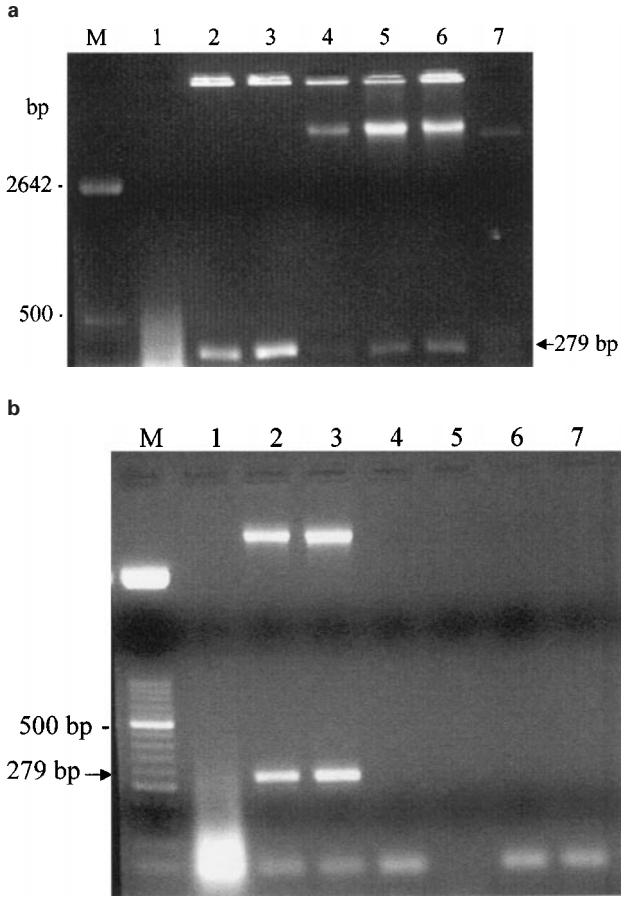

Figure 5 (**A**) Detection of HIV-1 RNA in culture supernatant of HBMEC in the early days following infection. Total RNA was prepared from culture supernatant, and RT–PCR performed as described. Lanes 1–7 are respectively: 1. negative control, 2: monocytes infected with HIV-1B (clade B, day 15 p.i.), 3: monocytes infected with HIV-1D (day 15 p.i.), 4: HBMEC infected with HIV-1B (day 3 p.i.), 5: HBMEC infected with HIV-1D (day 3 p.i.), 6: HBMEC infected with HIV-1D (day 5 p.i.), 7: HCN-1A infected with HIV-1B (day 5 p.i.). M is a 50 bp ladder (Roche Molecular Biochemicals). (**B**) HIV RT–PCR on HBMEC and HCN samples collected at 12 days p.i.

independent mechanisms, respectively (Steffan *et al*, 1992; Ray *et al*, 1998). There are conflicting reports as to whether HIV-1 can productively infect brain endothelial cells. It has been suggested that brain endothelial cells can support HIV-1 replication (Moses *et al*, 1993; Moses and Nelson, 1994) while others have failed to replicate these results (Nottet *et al*, 1996; Fiala *et al*, 1997).

HIV-1 can successfully infect its target cells only if they express viral receptors and/or co-receptors. It has been shown that brain-derived endothelial cells do not express CD4 receptor (Wiley *et al*, 1986; Harouse *et al*, 1989). However if HIV co-receptors are present in these cells, a CD4-independent HIV infection could occur (Harouse *et al*, 1989; Tateno *et al*, 1989; Li *et al*, 1990). Therefore, we studied the expression of CXCR4 and CCR5 chemokine receptors in human brain endothelial cells. In the current study, using a human brain endothelial cell line, we demonstrate the presence of CXCR4 and CCR5 co-receptors by RT–PCR but not by flow cytometry. It is possible that the CXCR4 and CCR5 genes are expressed and translated but that the level of proteins present on the cell surface is below the detectability threshold for flow cytometry. Also, levels of surface protein expression might be lower in cell lines compared to primary culture, and could be further decreased with cell passages and prolonged culture. Alternatively, it is possible that CXCR4 and CCR5 mRNA are expressed but that a post-transcriptional block occurs preventing translation, or perhaps more distally, movement, to the cell surface.

Our RT–PCR results showing more copies of CXCR4 than CCR5 agree with other published reports (Feil and Augustin, 1998; Berger *et al*, 1999). Feil and Augustin (1998) show by RT–PCR and immunohistochemistry high expression of CXCR4 in human umbilical vein endothelial cells, human dermal microvascular endothelial cells, and bovine aortic endothelial cells, but no expression of CCR5 in any of those cells. We also demonstrate high expression of CXCR4 on brain endothelium, but expression of CCR5 was often not detected unless cells were treated with TNF-α for 24 h prior to RNA extraction. This shows that TNF-α upregulates CCR5 mRNA levels. However, even with TNF-α treatment, the copy number of CCR5 mRNA was very low compared to CXCR4. These data are similar to a recent report showing a fivefold higher expression of CXCR4 compared to CCR5 in primary cultures of brain microvascular endothelium (Berger *et al*, 1999). Detection of CCR5 on brain tissue using immunostaining has been reported, but other investigators could not find CCR5 on brain endothelium of both patients with HIV encephalitis and non-encephalitic HIV infected controls (Sanders *et al*, 1998).

Expression of even a few molecules of a co-receptor per cell could be sufficient to allow viral entry. However, our experiments using two dual-tropic HIV-1 primary isolates fail to demonstrate *in vitro* infection of a human brain endothelial cell line. The positive p24 antigen results obtained in the early days after infection are most likely due to residual infection from the viral inoculum, as most samples collected after 5 days post infection are negative by RT–PCR and have no detectable p24 antigen or RT activity. Because TNF-α upregulates CCR5 RNA levels in brain endothelial cells, it is possible that TNF-α treatment of those cells could increase their susceptibility to HIV infection. However, treatment of brain endothelial cells with TNF-α does not increase susceptibility to infection. Our results are in agreement with those of Nottet and colleagues (1996) and Fiala *et al* (1997) who show that human brain endothelial cells are not

susceptible to infection by M-tropic and neurotropic HIV-1 isolates, despite the high titer of virus used. Finally, we and others (Moses *et al*, 1993; Moses and Nelson, 1994; Poland *et al*, 1995; Nottet *et al*, 1996), find that HIV infection does not induce a cytopathic effect on brain endothelial cells in culture.

In contrast to our results, some investigators have reported HIV infection of human brain endothelial cells (Moses *et al*, 1993; Moses and Nelson, 1994). In these studies, primary cultures of brain endothelial cells are infected with three HIV-1 isolates (one T-tropic, one M-tropic and one dual-tropic) and infectivity is evaluated based on p24 antigen staining of fixed cells starting at day two post infection. They demonstrated productive infection only with the T-tropic laboratory isolate (LAV). In contrast, a number of studies have shown that HIV infected cells in the brain are mainly of the monocytes/macrophages lineage (Wiley *et al*, 1986; Koenig *et al*, 1986; Price *et al*, 1988; Takahashi *et al*, 1996). Furthermore, studies in primates showed neurovirulent SIV strain was M-tropic (Anderson *et al*, 1993; Mankowski *et al*, 1994; Edinger *et al*, 1997). These data suggest that infection of brain endothelial cells, if possible, would be more likely by M-tropic or dual-tropic HIV isolates that infect macrophages and/or monocytes, not T-tropic isolates. Finally, in the current study we also observe positive p24 antigen by ELISA and positive RT–PCR up to day five post infection. However, our interpretation is that this is likely to be residual infection because no sample show detectable reverse transcriptase activity, most samples are negative for p24 antigen and by RT–PCR after day five post infection, and PCR does not detect proviral DNA in the infected cells.

Human neurons and most neuronal cell lines do not express the CD4 molecule (Harouse *et al*, 1989). We did not detect any CCR5 or CXCR4 on the human cortical neuronal cell line HCN-1A, using both RT–PCR and flow cytometry. However, other studies have shown the presence of HIV co-receptors on neurons, notably CXCR4 (Hesselgesser *et al*, 1997; Lavi *et al*, 1997; Sanders *et al*, 1998; Vallat *et al*, 1998; Klein *et al*, 1999) and CCR5 (Rottman *et al*, 1997; Klein *et al*, 1999). Most studies where the presence of CC and CXG chemokine receptors on neurons have been reported utilize immunohistochemistry (Rottman *et al*, 1997; Hesselgesser *et al*, 1997; Lavi *et al*, 1997; Sanders *et al*, 1998; Vallat *et al*, 1998; Klein *et al*, 1999), whereas we have used RT–PCR and flow cytometry. The difference in these techniques could contribute to the discrepancies observed between these studies. These discrepancies may also be due to the type of samples used in the different studies. Most studies where CC and CXC chemokine receptors are demonstrable utilize fresh tissue from brain biopsies (Lavi *et al*, 1997) or primary cultures of freshly isolated human neurons (Vallat *et al*, 1998; Klein *et al*, 1999), while in our studies, a stable neuronal cell line was used. This could be relevant because maintenance of the neuronal phenotype *in vitro* may present special problems, separate from the general caveats and limitations of cell culture, as discussed below. After passage, it is possible that cell lines will not express the HIV-1 chemokine receptors on their surface, or the level of surface protein expression might be very low and undetectable by the techniques used. In this regard, we have sporadically observed, in early passage HCN-1A, limited CXCR4 expression (data not shown).

Neuronal cell death is believed to be the direct cause of AIDS dementia, and this has prompted many laboratories to study the ability of HIV to infect human neurons (Harouse *et al*, 1989; Li *et al*, 1990; Sharpless *et al*, 1992; Truckenmiller *et al*, 1993; Mizrachi *et al*, 1994; Ensoli *et al*, 1995). The neuronal cell line SK-N-MC (Li *et al*, 1990) and primary human neuroblasts (Ensoli *et al*, 1995) were found to be susceptible to productive infection by HIV-1 *in vitro*, suggesting that immature and undifferentiated neurons may be susceptible to HIV infection (Sharpless *et al*, 1992; Truckenmiller *et al*, 1993; Ensoli *et al*, 1995). Other studies have shown evidence of low level infection of the human cortical neuronal cell line HCN-1A by HIV-1 when treated with nerve growth factor and fibroblast growth factor (Sharpless *et al*, 1992; Truckenmiller *et al*, 1993; Mizrachi *et al*, 1994). In contrast, *in situ* analyses and PCR do not reveal any evidence of HIV infection of neurons in the brain of AIDS patients, even in patients with HIV encephalitis and AIDS dementia (Wiley *et al*, 1986; Price *et al*, 1988; Takahashi *et al*, 1996; An *et al*, 1999). The data presented herein, showing a lack of productive infection of HCN-1A, as assessed by p24 antigen, RT activity, RT–PCR and DNA PCR is in agreement with the observation that no study to date has confirmed HIV-1 infection of primary neuronal cultures. Finally, the majority of studies performed *in vivo*, demonstrate that HIV does not infect neurons (Wiley *et al*, 1986; Price *et al*, 1988; An *et al*, 1999).

Our experiments examining co-receptor expression and HIV infectivity in both endothelium and neurons have utilized established cell lines. Comparing primary cultures to passaged cells can be difficult, and extrapolating from cell culture observations to *in vivo* processes even more problematic. Thus, some of the disparities among published data in this field could be due to variation in cell lot, number of passage and state of cell differentiation. In addition, endothelial cells display morphologic and functional heterogeneity depending on vessel size as well as specific organ specialization. While many but not all highly differentiated functions and markers of endothelial cells can be maintained with passage and time in

culture, the neuronal phenotype appears to be more variable. Because mature neurons are terminally differentiated and therefore not proliferative, many cultures utilize fetal neurons or established cell lines to obtain sufficient numbers of cells. It is not clear how closely these cells express features of mature neurons.

In summary, the susceptibility of brain-derived endothelial and neuronal cells to HIV infection remain controversial. The results from this study suggest that neither brain endothelial cells nor neurons are susceptible to *in vitro* infection by HIV-1. Because of the caveats regarding the use of cell lines discussed above these data are not conclusive. However, our results, in conjunction with many published reports, some utilizing fresh tissues, suggest that other cell types in the CNS, mostly macrophages and microglia, are the primary focus of HIV-1 infection in the brain. Further studies are required in order to determine how HIV invades the brain, the direct and/or indirect involvement of HIV in neuronal cell damage, and to elucidate the mechanism of neuronal cell death that underlies AIDS dementia.

Materials and methods

Cell culture and viral isolates

HBMEC were purchased from Cell Systems (Seattle, WA, USA) and HCN-1A from ATCC (Manassas, VA, USA). HBMEC were seeded on to 65 mm tissue culture plates coated with attachment factor (Cell Systems) and cultured in CS-C complete media (Cell Systems) according to the manufacturer's instructions. HCN-1A cells were seeded on to 100 mm culture plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2.2 g/l sodium bicarbonate, 10% fetal bovine serum (FBS, Hyclone) and 4 mM L-glutamine (Gibco, Rockville, MD, USA). For both cell types, culture medium was changed twice per week and confluent cells subcultured at a split ratio of 1 : 3 and 1 : 2 for HBMEC and HCN-1A, respectively. HBMECs were subcultured with the Passage Reagent Group solutions (Cell Systems) and HCN-1A with trypsin-EDTA (0.25% trypsin, 0.03% EDTA).

HOS.CCR5 and HOS.CXCR4, cell lines expressing CCR5 and CXCR4 respectively, and HOS.-pBABE-puro were from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. They were grown in DMEM containing 10% FBS, 1.0 μg/ml puromycin. All cell lines were maintained in an incubator (37°C, 5% CO_2). Normal human blood was obtained from the Sylvan Goldman Oklahoma Blood Institute (Oklahoma City, OK, USA). Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood by the standard Ficoll-Hypaque method. Monocytes/macrophages were purified from fresh PBMC by the 5-day-adherence method (Perno and Yarchoan, 1993) in 25 or 75 cm^2 flasks, and maintained in RPMI 1640 supplemented with 10% FBS, 10% normal human serum (Type AB), 5 U/ml human interleukin-2 (Boehringer Mannheim, Indianapolis, IN, USA) and 1% antibiotic/antimycotic solution (Gibco).

Two primary HIV-isolates, BZ167 (clade B) and UG001 (clade D), were used in this study. Their origin have been described elsewhere (Benton *et al*, 1999). These isolates, initially T-tropic, were adapted and expanded in monocyte/macrophages. Briefly, HIV-1 was used to infect freshly prepared monocyte/macrophages at a concentration of 200 $TCID_{50}$/ml. Viral inoculum was washed away after 48 h, and infection monitored from day 6 by syncytia formation, RT activity and p24 antigen capture assay. Expansion of the two isolates was performed by subsequent infection of freshly prepared monocyte/macrophages with culture supernatant at the peak of RT activity. Isolates used in this study had been expanded three times and could easily infect both monocyte/macrophage and PBMC from a variety of human donors.

CXCR4 and CCR5 RT–PCR

Confluent cells were washed twice with PBS and detached from plates by scraping with a Cell Lifter (Fisher). Total RNA was isolated from cells with the TRIZOL reagent (Gibco), as recommended by the manufacturer. Two micrograms RNA were reverse transcribed in a 20 μl reaction volume, according to the SUPERSCRIPT™ RT II (Gibco) protocol. Five microliters of cDNA were used in the PCR reactions (50 μl) with CXCR4- and CCR5-specific primers. Primers sequence and reaction conditions have been described elsewhere (Benton *et al*, 1999). PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA), and final PCR products analyzed by standard agarose gel electrophoresis. For each experiment, a control PCR reaction was done with β-actin primers using the same cDNA volume (5 μl). Sequence of the β-actin primers were as follows: 5′-CCAGCCAGGTCCA-GACG-3′ and 5′-CAGGCACCAGGGCGTGATG-3′ (respectively for the forward and reverse primers).

Flow cytometry

Confluent cells were washed twice with PBS and detached using either trypsin-EDTA (HCN-1A) or the Passage Reagent Group solutions (HBMECs). Monoclonal antibodies to CXCR4 (12G5, IgG2a) and CCR5 (2D7, IgG1) were obtained through the AIDS Research and Reference Reagent Program and used at the recommended concentration. A monoclonal antibody (419, IgG2a) specific for simian virus 40 large tumor antigen (Shearer *et al*, 1994) was used as control for non-specific antibody binding, at a similar concentration. Brain endothelial cells, neurons, HOS.CCR5 and HOS.CXCR4 cells were washed twice with PBS, and incubated with appropriate monoclonal antibodies (30 min at

4°C). Cells were then washed twice with PBS containing 1% BSA, 0.1% sodium azide, and incubated 30 min at 4°C with FITC conjugated goat anti-mouse immunoglobulin (Ig) (1 : 250 dilution). Cells were again washed twice as above, then fixed in a 1% paraformaldehyde solution, and analyzed immediately using a Becton-Dickinson Fac-Star Plus flow cytometer.

HIV-1 infection of brain endothelial cells and neurons

At the third culture expansion in monocyte/macrophage, the two dual-tropic HIV isolates were used to infect brain endothelial cells and neurons grown to 80% confluency. Culture supernatant from HIV infected monocyte/macrophage at the peak of RT activity, were filtered through a 0.45 μM filter, diluted 1 : 3 with culture medium, and used to infect HBMEC and HCN for 48 h. Viral inoculum was removed by extensive washing of cells (six washes) with serum-free media. Cells were resuspended in their respective culture media and maintained at 37°C, 5% CO_2. Starting at day 3 p.i. culture supernatant was collected every 3–4 days and tested for p24 antigen, RT activity and by RT–PCR. Some brain endothelial cells were sub-cultured by trypsinization at 3 days p.i., and culture supernatant regularly collected and tested as above.

p24 antigen capture ELISA, RT assay, HIV RT–PCR and DNA PCR

A commercial ELISA kit from Coulter (Miami, FL, USA) was used to measure p24 antigen in culture supernatant, according to the manufacturer's instruction. The RT assay method was essentially as described by Pyra *et al* (1994). Briefly, viral particles were pelleted from filtered culture supernatant (1 ml) by ultracentrifugation (70 000 × g; 90 min). The pellet was then lysed (final volume of the lysate was 30 μl) and 3 μl of the lysate used in an RT-mediated cDNA synthesis, with bacteriophage MS2 RNA as template. cDNA produced was then selectively amplified with MS2 specific primers. Amplicons were then hybridized to biotin and digoxigenin-labeled probes and detected by ELISA.

For RT–PCR, RNA was extracted from culture supernatant of infected cells using the TRIZOL reagent or QIAamp Viral RNA kit (Qiagen, Valencia, CA, USA). Five micrograms of RNA were reverse transcribed as described above using random primers. Five microliters of cDNA were amplified (50 μl total reaction volume) using standard protocols (*Taq* DNA polymerase, Gibco). PCR primers were from the Gag region of HIV-1, and their sequence was as follows: 5′-TAGAAGAAATG-ATGACAGCATG-3′ (nucleotides 1370–1391) and 5′-TCCCTAAAAAATTAGCCTGTC-3′ (nucleotides 1648–1628) (respectively for the forward and reverse primers). The PCR cycle was as follows: 94°C for a 5 min initial denaturation followed by 45 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final extension of 7 min at 72°C. The samples were then cooled to 7°C. To test for the presence of viral DNA in cells, DNA was extracted from infected cells at 12–17 days post infection using *Wizard*™ Genomic DNA Purification Kit from Promega (Madison, WI, USA). Five to 10 μg DNA was amplified as above. Final PCR products were analyzed by agarose (2%) gel electrophoresis.

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