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Comparisons of HIV-1 viral sequences in brain, choroid plexus and spleen: Potential role of choroid plexus in the pathogenesis of HIV encephalitis

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Possible mechanisms of HIV transmission to the brain include direct viral infection of cerebral endothelium and hematogeneous dissemination of viral-infected lymphocytes and monocytes. Cerebrospinal fluid dissemination from a primary infection of choroid plexus (CPx) is an alternative mechanism supported by recent studies in our laboratory. We showed that HIV-infected asymptomatic patients as well as AIDS patients have HIV infection of the CPx; the cell types so infected included stromal monocytes and dendritic cells. To further explore the potential role of CPx in the pathogenesis of HIV encephalitis, we analyzed HIV sequences from brain, CPx, and spleen of four AIDS patients by extracting DNA from paraffin sections and amplifying the V3 region of the HIV *env* gene by PCR. Several different clones from each tissue were characterized. We found that viruses from the brain and spleen grouped into two distinct clusters, while viruses of the CPx contained viral strains that were a mixture of those found in the brain or spleen. Net charge analysis of the V3 tip region showed that the brain viral sequences had fewer positive charges than blood viral sequences. Our results support the hypothesis that CPx may be one of the sites where HIV-1 gains access to the brain from the blood and therefore contains viruses that are of both genotypes. *Journal of NeuroVirology* (2000) **6,** 498–506.

Keywords: HIV; choroid plexus; brain; viral sequences

Introduction

Human immunodeficiency virus (HIV) infects the central nervous system (CNS) soon after the initial systemic infection. The virus can be isolated from the cerebrospinal fluid (CSF) within the first few months after seroconversion and during the period of clinical latency (Ho *et al*, 1985; Goudsmit *et al*, 1986; Chiodi *et al*, 1992). Actual HIV encephalitis with productive viral infection of the brain is rare prior to the onset of immunosuppression and the acquired immunodeficiency syndrome (AIDS), although HIV DNA can be amplified from brains of HIV-infected asymptomatic patients (An and Scaraville, 1997; Sinclair *et al*, 1994; Di Stefano *et al*, 1996). The isolated viruses are macrophage-tropic (Reddy *et al*, 1996; Brew *et al*, 1996) and viral reproduction is confined to microglia and monocytes rather than neurons or glia (Koenig *et al*, 1986; Wiley *et al*, 1986). HIV encephalitis is closely linked to a dementing illness variably known as AIDS dementia complex or HIV-associated dementia, a disorder that can affect 30% or more of AIDS patients at the time of death (Snider *et al*, 1983; Navia *et al*, 1986; Report of a working group of the AAN AIDS task force, 1991).

Specific information concerning the mode and site of viral entry into the CNS is lacking. It is not clear whether viral dissemination takes place as free virus, HIV-infected T lymphocytes, or HIV-infected monocytes. Actual endothelial infection is rarely found *in vivo* although it is prominent in experimental retroviral infection (Mankowski *et al*, 1994), and detected in human endothelium *in vitro* (Moses *et al*, 1993; Poland *et al*, 1995; Gilles *et al*, 1995). Different factors could facilitate free or intracellular viral entry into the CNS in AIDS patients. These

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include breakdown of the blood-brain barrier (Petito and Cash, 1992; Power *et al*, 1993), the high incidence of opportunistic infections in the CNS (Sharer, 1992), the presence of CXCR4 HIV chemokine coreceptors on endothelium (Lavi *et al*, 1997), and cytokine-induced alteration of endothelial adhesion molecules (Nottet *et al*, 1996).

The choroid plexus (CPx) is an alternative site of viral entry. In contrast to brain parenchyma, this structure has permeable capillaries that reside outside of the blood-brain barrier (BBB) (Tennyson *et al*, 1968) and a normal stromal compliment of blood-borne lymphocytes and monocytes (Hickey *et al*, 1992; Maxwell *et al*, 1992; Matyszak *et al*, 1992). These characteristics may enhance the potential role of CPx in the hematogeneous dissemination of infectious agents, including retroviruses such as feline immunodeficiency virus, visma and simian immunodeficiency virus (Bell *et al*, 1993; Chakrabarti *et al*, 1991; Beebe *et al*, 1994). HIV also infects the CPx. Harouse *et al* (1989) found that a sub-population of human CPx stromal cells sustain productive HIV infection *in vivo*. In addition, Bagasra *et al* (1996) found HIV infected CPx endothelial cells by a combination of *in-situ*-polymerase chain reaction (IS-PCR) and reverse transcription (ISH-PCR). We found that almost 50% of CPx from AIDS patients, as well as 25% of HIV-infected asymptomatic patients, contain HIV-infected cells by immunohistochemistry (Falangola *et al*, 1995; Petito *et al*, 1999). In a subsequent study, we showed that HIV-infected cells could include antigen-presenting cells with a prominent dendritic morphology (Hanly and Petito, 1998). We hypothesized that the infected CPx dendritic cell, previously identified in human material by Serot *et al* (1997), function in a similar fashion as Langerhans cells of the epidermis and dendritic cells of systemic organs. This includes the ability to harbor low-grade persistent HIV infection during the prolonged period of clinical latency (Spiegel *et al*, 1992; Pantaleo *et al*, 1993).

If the CPx is a site of HIV dissemination, we hypothesize that it would contain an admixture of systemic and brain viral strains. Accordingly, we selected four AIDS cases with HIV-infected cells in the CPx and analyzed proviral DNA sequences from CPx, brain, and spleen (to represent systemic virus) for their genetic relationships in the V3 region of HIV envelope glycoprotein. We used DNA extracted from formalin-fixed paraffin-embedded tissue since frozen materials from all three tissue-sites were unavailable for these four AIDS cases. We found that CPx-derived viruses represent a mixture of splenic and brain sequences, suggesting that this structure may provide a site for hematogeneous dissemination with subsequent spread to the brain. A preliminary report has been published (Petito *et al*, 1998).

Results

HIV sequences from brain, CPx and spleen

Alignment of the 220 bp region flanking the V3 loop region from different tissues from the same patients revealed a tissue specific distribution (Figure 1). The viral sequences from the brain isolates clustered and were more related to each other than to those from the CPx or spleen. Similarly, the viral sequences from the spleen isolates clustered and were more related to each other than CPx and brain. In contrast, the CPx viral sequences contained a mixture of sequences found in isolates from both the brain and spleen. For example, in patient 122, a 'G' was found at nucleotide position 24 in all the brain viral clones analyzed, but an 'A' was found in the same position in all the spleen isolates. In contrast, one clone from the CPx contained an 'A' and two clones contained a 'G', suggesting that viruses in the Cpx represented a mixture of those that were found in the brain and in the blood (Figure 1). In patient 1404, brain isolates contained 'CC' at nucleotide positions 29 and 30, whereas splenic isolates had 'AA', and CPx isolates had both. Similar clustering of viral sequences was observed in patients 266 and 407. The spleen viral sequences formed a cluster and were distinct from the brain viral sequences. The CPx isolates were intermediate between the brain and spleen isolates, and contained signature sequences of both the brain and spleen isolates.

Homology analysis of the HIV sequences

To further confirm the genetic relationship among the viral isolates from different tissues of the four patients, we performed pair-wise sequence analysis using the GCG sequence analysis program to calculate sequence homology. Sequences from all tissues were compared against each other, and sequences restricted to a particular tissue, such as brain, CPx, and spleen, were compared with each other. The resultant homologies ranged from 84.0% to 98.9% (Table 1).

In most cases, the sequence homology was highest among viruses obtained from the same tissue. In all 12 samples analyzed, viral sequence homology within the same tissue was greater than 89.3%, with an average of 95.1%. In contrast, the mean sequence homology between brain and spleen isolates was the lowest in all samples (Table 1). Two patients (266 and 407) had CPx viral sequences that were more homologous to brain viral sequences than to spleen viral sequences, whereas for the other two patients (266 and 407), the CPx viral sequences were more homologous to spleen than to brain viral sequences (Table 1). If we assume that higher homology represents a closer evolutionary relationship, viruses from the brain and spleen showed the farthest relationship whereas viruses from CPx had relatively closer relationships to viruses either from brain and spleen.

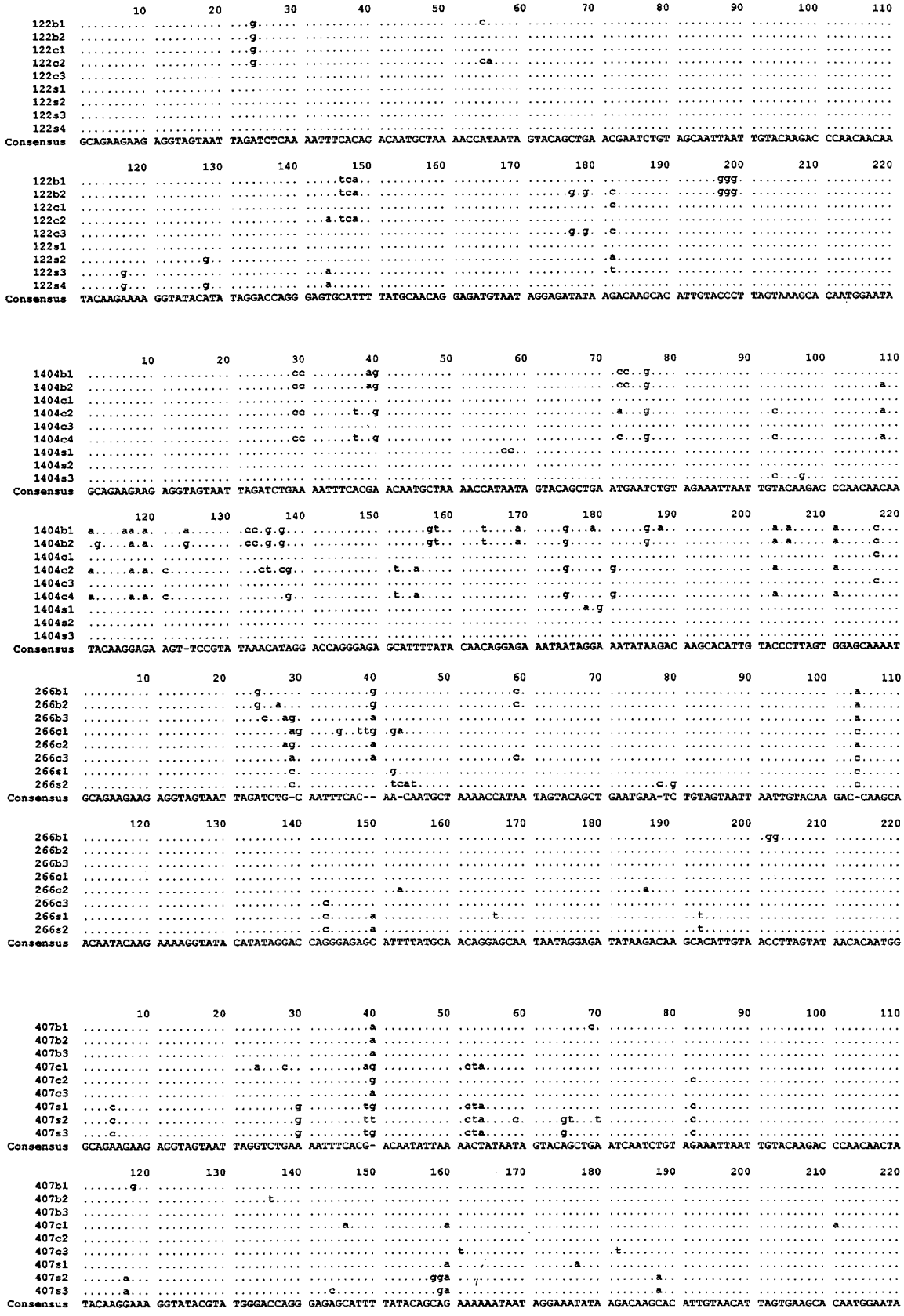

Figure 1 Alignment of DNA sequences of the HIV envelope V3 region obtained from brain, CPx, and spleen tissues of the four patients analyzed. The consensus sequence of each patient is shown at the bottom. S represents spleen sequences; C-CPx; B-brain.

Phylogenetic analysis of intra-patient sequence sets
We confirmed the above results using phylogenetic analysis of the various viral sequences obtained from brain, CPx, and spleen (Figure 2). The phylogenetic analysis of all four patients showed the spleen viral sequences clustered into one branch and the brain viral sequences into another. The CPx viral sequences were found in both the brain and spleen clusters. As found by sequence homology, some of the CPx viruses were more related to the brain isolates (patients 266 and 407) while others were more related to the spleen isolates (patients 122 and 1404). Lastly, no tissue specific signature sequence was identified (Brew *et al*, 1996; Korber *et al*, 1994; Hughes *et al*, 1997).

Table 1 Homology analysis of the HIV V3 sequences from brain/CPx/spleen tissues.

Sample no.	*122 (%)*[a]	*1404 (%)*	*266 (%)*	*407 (%)*
Brain *vs* spleen	95.3±0.7	84.0±0.6	90.5±0.5	91.7±1.4
Brain *vs* CPx	96.3±1.0	85.4±0.7	94.4±1.0	95.8±2.0
CPx *vs* spleen	97.6±1.2	90.6±4.3	91.5±0.8	92.2±1.5
Brain *vs* brain	98.0±0.0	96.0±0.0	91.5±1.2	97.0±0.0
Cpx *vs* CPx	98.0±1.7	89.3±4.5	94.7±1.4	94.3±1.5
Spleen *vs* spleen	98.9±0.4	94.7±0.6	94.0±0.0	94.7±0.6

[a]% homology are shown as average values of all the sequences analyzed.

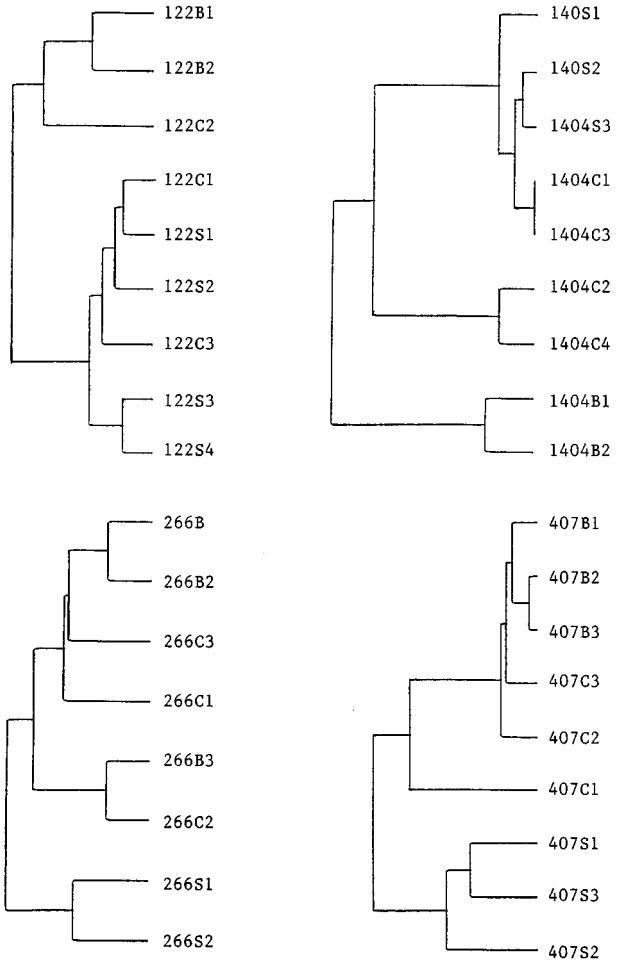

Figure 2 Phylogenetic trees constructed by the UPGMA method. The sequences obtained from brain, CPx, and spleen of the same patient were aligned with the Clustal W program and analyzed by using the evolution program in the GCG package. The number represents the patient number and B1 represents brain HIV sequence 1; C-CPx; B-brain etc.

Net charge of the V3 tip region
We determined the relative charge of the V3 domain since this property has been suggested to influence viral function and correlate with viral phenotype, including fusion capacity and tropism (Callahan *et al*, 1991; Fouchier *et al*, 1992; Korber *et al*, 1994). Since we did not have fresh frozen samples of all three tissues available for viral isolation to study their biological properties, we calculated the net charge of a 16-amino acid region of V3 loop, which included the 4-amino acid tip (GPGR and its analog) flanked by 6 amino acids from both N- and C-terminal sides from our brain, CPx, and brain viral sequences. The sequence distribution pattern based on the net positive charge of the V3 tip region showed that the brain-derived sequences were less positive when compared to the spleen-derived sequences, thereby suggested that the brain-derived sequences are more likely to be macrophage-tropic (Figure 3). The net positive charge of the V3 region of the CPx-derived sequences fell in between the brain- and blood-derived sequences, again suggest-

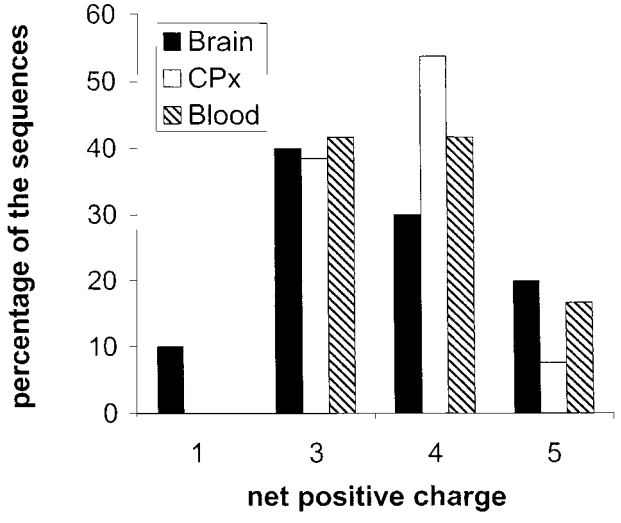

Figure 3 Net positive charge of the HIV envelope V3 tip region sequences obtained from brain, CPx and spleen tissues of the four patients. The net charge of 16-amino acids at the tip of the V3 loop, as described in the text, was calculated. All the sequences analyzed in this study were grouped by the tissue source. The number at the x-axis represents the net positive charge of the amino acids sequence. The percentage represents the sequence distribution based on the net positive charge in each group.

ing that there was an admixture of brain and systemic viruses in the CPx.

Discussion

The present study examined viral sequences obtained from brain, CPx, and spleen (representing blood isolates) from four patients with AIDS. On the basis of sequence homology analysis and phylogenetic analyses, our studies confirmed that brain and splenic sequences are distinct from each other and form distinct clusters in each patient. This observation was found in all four patients and confirmed by different sequence analysis methods. It is in agreement with several studies (Ball *et al*, 1994; Korber *et al*, 1994; Wong *et al*, 1997), which also found that the V3 loop sequences clustered according to brain versus blood isolates. However, such clustering was not as obvious in other reported studies (Beebe *et al*, 1994; Reddy *et al*, 1996; Brew *et al*, 1996; Hughes *et al*, 1997).

Our results support our hypothesis that the CPx may be important in the neuropathogenesis of HIV encephalitis and a site of viral entry since it contained mixtures of brain and blood isolates. Viral sequences from the CPx did not form clusters distinct from those in brain and in spleen in any of the four patients. Rather, phylogenetic analysis revealed that the CPx viral sequences are related to both the brain and spleen subgroups. In all four patients studied, the CPx contained admixtures of genetic sequences of both brain and splenic viruses. In two, CPx viral sequences were more homologous to the brain than to the spleen whereas in the other two patients, the CPx viral sequences were more homologous to the spleen than to the brain. Analysis of the net charge of the V3 region also supported the concept that the CPx viruses represent admixtures of those from brain and systemic sources.

There are several limitations to this initial analysis. We studied only a small number of patients and a limited number of viral sequences; it is not possible to determine whether these are representatives of the entire viral repertoire. Additional studies using a larger number of patients and analyzing a larger number of clones from the entire *env* gene are needed. Post-mortem autolysis and tissue fixation could affect the PCR amplification and prevent amplification of less abundant viral sequences. We reduced this complication as much as possible by limiting the study to those cases with post-mortem intervals of 12 h or less, and maintaining formalin-fixation periods between 10 and 12 days. Lastly, we were not able to characterize the biological properties of these viruses, including cellular tropism and syncyctium forming ability, for which fresh or fresh-frozen material is required for viral isolation.

The presence of brain-related viral sequences in the CPx is consistent with CPx infection of monocytes/macrophages and is in concert with our prior study (Falangola *et al*, 1995; Hanly and Petito, 1998). However, the cell tropism of the splenic-related viral isolates in the CPx is not clear. The tissues for our current study were obtained from end-stage AIDS patients, in whom most blood-derived viral isolates are lymphotropic (Connor *et al*, 1997; Lu *et al*, 1997; Speck *et al*, 1997). For that reason, we suggest that they represent T cell tropic viruses infecting CPx T lymphocytes. The presence of T cell-tropic virus in the CPx offers a possible mechanism for these infected cells to enter brain via the CSF. This would allow them to directly damage brain neurons, which express high levels of the T-tropic HIV chemokine co-receptors (Hesselgesser *et al*, 1997, 1998; Lavi *et al*, 1997).

Prior studies have examined the potential for HIV strain and quasispecies specificity to confer macrophage tropism, neurotropism, and neurovirulence as measured by the development of HIV-associated dementia (Callahan *et al*, 1991; Fouchier *et al*, 1992; Korber *et al*, 1994). All agree that brain-derived viruses are macrophage-tropic when they were cultured *in vitro*. Korber *et al* (1994) reported that the brain-derived viral sequences had a tendency towards negative or neutral charge compared to the blood-derived viral sequences. This correlates with the charges differences between macrophage-tropic and non-macrophage-tropic isolates found by Fouchier *et al* (1992). Our net charges distribution pattern in brain *versus* spleen is consistent with these studies. These results suggest that the brain-derived and part of the CPx-derived sequences in this study may represent macrophage tropic viruses *in vivo*. The presence of any brain-specific motifs is controversial, and our analysis of the viral sequences in the current study did not identify any brain specific motif. This is in support of other studies, which also failed to detect associations between viral sequences and clinical staging (Koenig *et al*, 1986) or HIV-associated dementia (Brew *et al*, 1996). In contrast, some investigations suggest that there are brain-specific motifs (Korber *et al*, 1994); a relationship between brain isolates and clinical stage of AIDS (Brew *et al*, 1996; Korber *et al*, 1994; Keys *et al*, 1993); and specific viral sequences associated with HIV dementia (Power *et al*, 1994). Lastly, brain isolates displaying regional variability have been found in some studies (Cunningham *et al*, 1997; Fujimura *et al*, 1997) but not others (Koenig *et al*, 1986); and none has correlated specific signatures with specific brain regions.

Although our present study supports the hypothesis that CPx may be an important site for HIV brain infection, it does not rule out other mechanisms of viral entry. Direct endothelial infection develops in experimental retroviral infection (Mankowski *et al*,

1994) and thus, early viral entry by this pathway cannot be excluded. HIV-infected T lymphocytes could also be responsible, especially since activated T lymphocytes normally traverse the blood-brain barrier to a limited extent (Hickey *et al*, 1992). If they are involved, there may be a need for an intra-CNS viral mutation from a lymphotropic to a macrophage-tropic variant to allow infection of brain monocytes. Viral-infected monocytes also could enter the brain, either as a result of an altered blood-brain barrier, which does occur in many AIDS patients, an opportunistic brain infection, or altered cytokine environment that increases or reduces endothelial adhesion molecules.

Materials and methods

Patients and tissues

The four AIDS patients (No. 266, 407, 1404, 122) were men with an age range between 28 and 42 years, and a post-mortem interval range between 5 and 12 h (Table 2). Brain tissues and CPx from the patients were fixed for 10–12 days in 10% buffered formalin, serially sectioned and 13 areas selected for processing and microscopic examination as previously described (Petito *et al*, 1986). The choroid plexus and at least one section of brain (basal ganglia) were prepared for HIV gp41 immunoreactivity study using the avidin-biotin complex technique (Vector Laboratories, Carpenteria, CA, USA). Monoclonal antibodies directed against HIV gp41 (Genetics System, Seattle, WA, USA) were used at a 1:750 dilution. Patient 266 had HIV encephalitis manifested by the characteristic microglial nodules, multinucleated cells and HIV gp41 immunoreactivity; patient 1404 had a small (1 cm) contralateral basal ganglia hematoma of unknown etiology; patient 407 had cryptococcaal meningitis; and patient 122 had small foci of gliosis in the basal ganglia, but multinucleated cells and immunoreactivity for gp41 were absent. All contained HIV-immunoreactive cells in the CPx. None had formal neuropsychological testing for AIDS dementia although this diagnosis was not made in any of the four, including the one with HIV encephalitis.

DNA extraction

Two 20 μm-thick paraffin tissue sections were placed in a 1.5 ml eppendorf tube and dissolved in 800 μl of xylene and 400 μl of ethanol. To avoid cross contamination between specimens, a new microtome knife was used for each block during sample preparation. The samples were vigorously vortexed for 1 min and microcentrifuged for 3 min to pellet the tissues. Absolute ethanol was added and the tubes vortexed. The tissues were then spun down in a microcentrifuge for 5 min. We digested the pellet in 100 μl of a detergent/protease mixture (1% NP40 or Triton X-100 plus 2.4 μl of 2.5 mg/ml protease K) at 55°C overnight. The digested tissues were centrifuged and the supernatants saved for further DNA extraction using ISOQUICK nucleic acid extraction kit (ORCA Research Inc., Bothell, WA, USA). The DNA was resuspended in 25 μl of RNase-free water. This entire procedure, including tissue sectioning and DNA analysis, was repeated at a later time to ensure that sequences obtained were not due to laboratory contamination.

PCR reaction

The PCR reagents were added to give the following final concentration in a volume of 50 μl: 10 mM Tris HCl (pH 8.3), 1.5 mM Mg++, 75 mM KCl, 40 pmol of each primer (Henv-1 5′-GTA TGA ATT CAA CTG CTG TTA AAT GGC AGT-3′ and Henv-2 5′-ATG GAA TTC ACT TCT CCA ATT GTC CCT CAT-3′)

Table 2 Clinical history and post-mortem examinations of patients in this study.

Patient no.	*Age/ PMI*	*Risk factors*[a]	*Clinical history*	*Systemic autopsy findings*	*Neuropathological examination*	*HIV gp41*[b]
122	22 year/ 12 h	homosexual	HIV infection; acute bacterial and fungal septicemia; toxic liver necrosis	severe hepatic necrosis, gastrointestinal candidiasis, disseminated intravascular coagulopathy	mild white matter gliosis	Brain: – CPx: +
1404	42 year/ 10 h	IVDA	hepatitis C and HIV infection; pulmonary tuberculosis	blunt facial and extremity trauma, cirrhosis, pulmonary tuberculosis	basal ganglia hematoma of uncertain etiology	Brain: – CPx: +
407	34 year/ 10 h	N/A	HIV infection and cryptococcal meningitis	acute pulmonary edema, meningitis	cryptococcal meningitis	Brain: – CPx: +
266	36 year/ 5 h	N/A	HIV infection; miliary tuberculosis; past history of syphilis and typhoid	miliary tuberculosis, HIV nephropathy	HIV encephalitis	Brain: + CPx: +

[a]Risk factors unknown in patients 407 and 266; [b]HIV gp41-immunoreactive cells; Abbreviations: PMI: post-mortem interval; HIV: Human Immunodeficiency Virus; AIDS: Acquired Immunodeficiency Syndrome; CPx: Choroid Plexus; N/A: Not available.

and 2.5 U Taq Polymerase (Hutto *et al*, 1996). Five micro-liters of extracted DNA were than added to the PCR mixture. The reaction was performed in a DNA thermal cycler (Perkin-Elmer) using the following conditions: the first cycle at 94°C for 3 min, 50°C for 1.5 min and 72°C for 2 min; followed by 35 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 2 min; for the last cycle, the 72°C elongation was extended to 10 min. Five micro-liters of the above first round PCR products were used as template for nested PCR using internal primer (Henv-3 5′-CGG AAT TCG CAG AAG AAG AGG TAG TAA TTA G-3′ and Henv-4 5′-TGT TCT AGA GTG TTA TTC CAT TGT G-3′). The PCR conditions used were the same as above.

Sequence analysis

The HIV envelope V3 region and flanking regions were amplified by PCR from three matched tissues (basal ganglia, CPx, and spleen) of the four AIDS patients. The amplified sequences were cloned using the PCR Vector pGEM-T kit (Promega, Madison, WI, USA). At least two clones from each tissue of the four patients were sequenced using a T7 sequencing kit by the dideoxynucleotide chain determination method (Sanger *et al*, 1997). The sequences from the same patient were compared against each other using the Bestfit program in the GCG package (Madison, WI, USA), and the average values are shown in Table 1. Following the alignment of the sequences with the Clustal W. program, the phylogenetic trees were reconstructed by using the UPGMA method (GCG package, evolution program).

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