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Short Communication

Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues

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The importance of astrocytes as a reservoir of human immunodeficiency virus type 1 (HIV-1) in the brain remains elusive. By combining immunohistochemistry, laser capture microdissection, and triple-nested *Alu*-PCR, we demonstrate integrated HIV-1 in astrocytes and macrophages isolated directly from autopsy brain tissues of HIV-1-infected subjects. The ability of HIV-1 to integrate in terminally differentiated astrocytes suggests a permanent reservoir of provirus in brain that will impact the development and likely success of strategies aimed at eradicating HIV-1. *Journal of NeuroVirology* (2006) **12,** 146–152.

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Unique immunological selection pressures and specific target cell populations within brain allow human immunodeficiency virus type 1 (HIV-1) to undergo adaptive changes and persist within the central nervous system (CNS). Subsequently the genetic evolution of HIV-1 in brain is distinct from that in lymphoid tissues, which directs compartmentalization of virus in the CNS (Gonzalez-Scarano and Martin-Garcia, 2005). Previous studies identified astrocytes and macrophage-lineage cells as target cells for HIV-1 infection in brain (Takahashi et al, 1996). By genetically analyzing HIV-1 sequences cloned from pure cell populations isolated from brain tissue, we have recently shown that HIV-1 can be further compartmentalized between astrocytes and macrophage-linage cells within the brain microenvironment (Thompson *et al*, 2004).

Macrophages and microglia are the most significant cellular reservoirs of HIV-1 in the brain (Gonzalez-Scarano and Martin-Garcia, 2005; Takahashi *et al*, 1996), and have a critical role in the neuropathogenesis of HIV-1 infection (reviewed in Gonzalez-Scarano and Martin-Garcia, 2005). In contrast, considerable debate surrounds the contribution of astrocytes to the viral reservoir. Astrocyte infection is non-productive and relatively infrequent, suggesting that infected astrocytes do not contribute significantly to CNS viral load (Gonzalez-Scarano and Martin-Garcia, 2005; Gorry *et al*, 2003). However, the abundance of astrocytes means that even a small percentage of non-productively infected cells may contribute significantly to the overall burden of HIV-1 DNA (Brack-Werner, 1999). Integration of HIV-1 DNA into the host cell chromosome is an obligate step in the

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virus replication cycle (Englund *et al*, 1995). Thus, although astrocyte infection leads to altered astrocyte functions (Brack-Werner, 1999; Gonzalez-Scarano and Martin-Garcia, 2005; Gorry *et al*, 2003), the potential for astrocytes to act as a viral reservoir ultimately depends on whether they contain integrated (proviral) HIV-1 DNA. Infection of cultured astrocytes can result in integration of HIV-1 (Gorry *et al*, 2003),

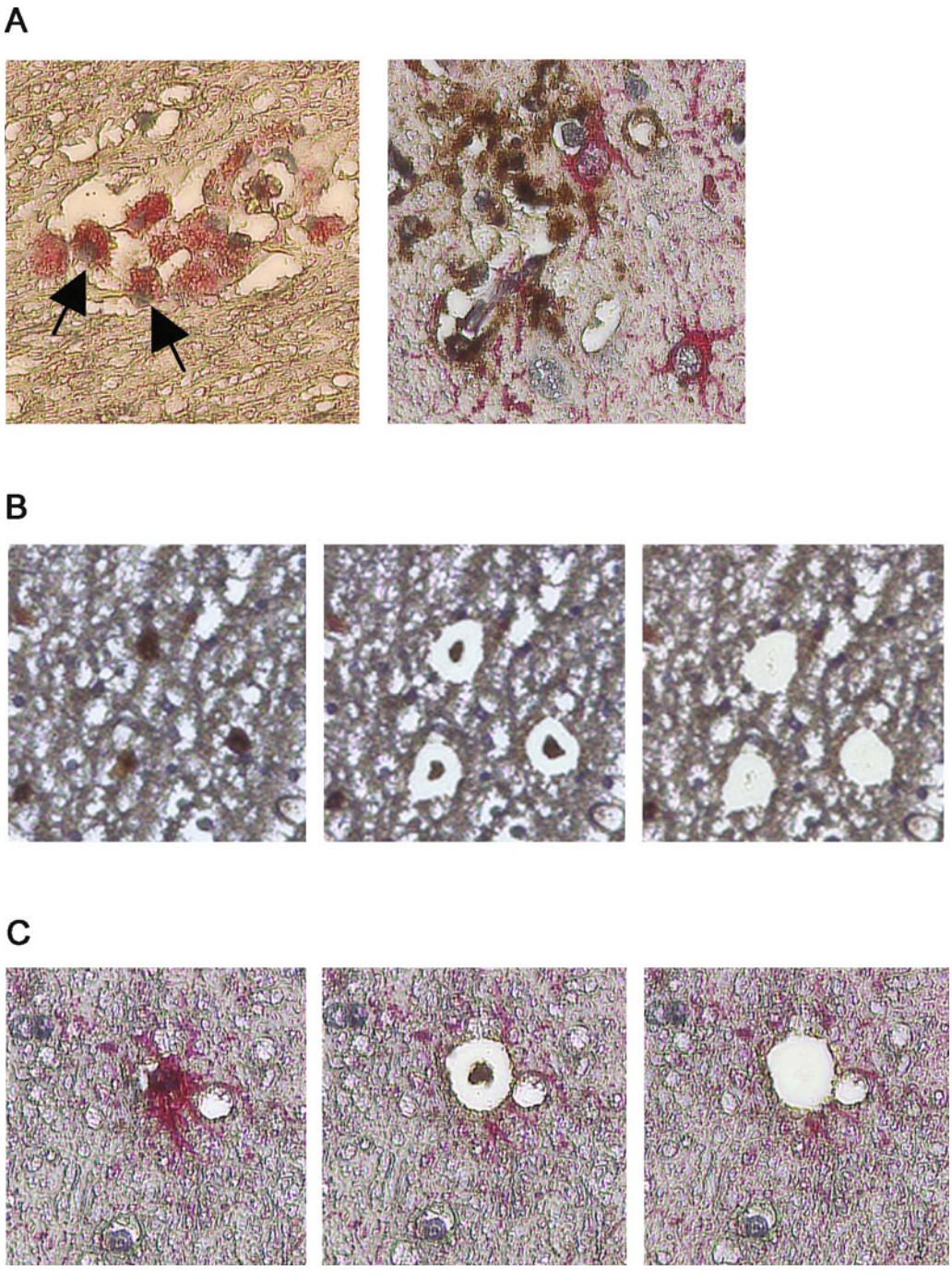

Figure 1 Cell identification and microdissection from formalin-fixed, paraffin-embedded human brain tissue. (**A**; *left panel*) Double IHC staining to detect CD68 in macrophage lineage cells (red) and HIV-1 p24 antigen (brown). Arrowheads indicate cells positive for both CD68 and HIV-1 p24 antigen. (**A**; *right panel*) Double IHC staining to detect GFAP in astrocytes (red) and CD68 in macrophage-lineage cells (brown). The micrographs shown are derived from uncoverslipped brain sections of patient 1. Similar results were obtained using tissue derived from the other 4 HIV+ cases and 2 HIV− controls, except that HIV-1 p24 antigen was not demonstrated in tissue sections of controls. (**B**) CD68 IHC to detect macrophage-lineage cells, and (**C**) GFAP IHC to detect astrocytes in uncoverslipped brain sections prior to laser capture microdissection (left panels), after ablation of cytoplasm and surrounding matter (middle panels), and after catapultion of nuclei (right panels). The micrographs shown are derived from brain sections of patient 1. Similar results were obtained using tissue derived from the additional 4 HIV+ cases and 2 HIV− controls. All images are ×640 final magnification.

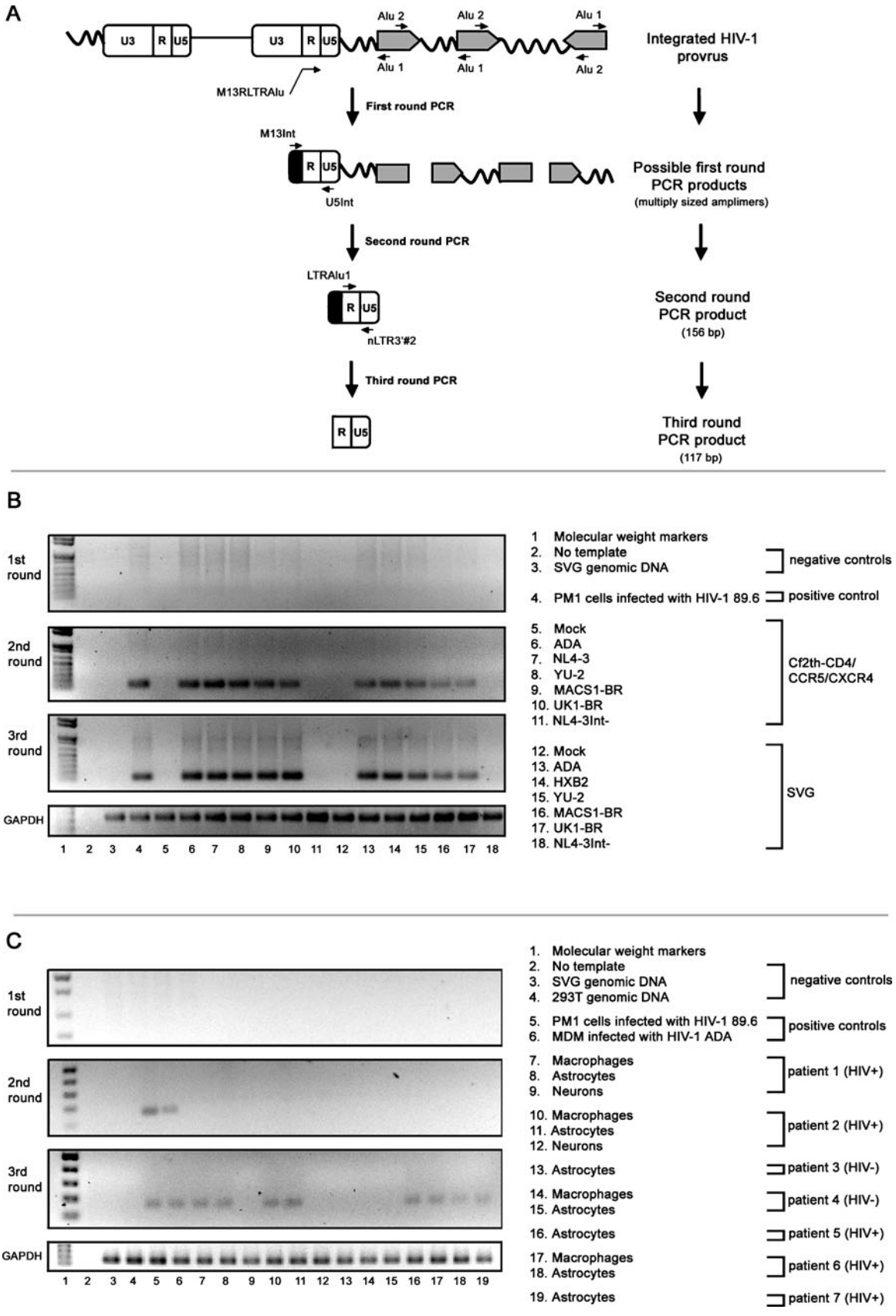
A
U3
R
U5
Alu 2
Alu 1
Integrated HIV-1 provrus
M13RLTRAlu
First round PCR
M13Int
U5Int
Possible first round PCR products
(multiply sized amplimers)
Second round PCR
LTRAlu1
nLTR3'#2
Second round PCR product
(156 bp)
Third round PCR
Third round PCR product
(117 bp)
B
1st round
2nd round
3rd round
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1 Molecular weight markers
2. No template
3. SVG genomic DNA
negative controls
4. PM1 cells infected with HIV-1 89.6
positive control
5. Mock
6. ADA
7. NL4-3
8. YU-2
9. MACS1-BR
10. UK1-BR
11. NL4-3Int-
Cf2th-CD4/ CCR5/CXCR4
12. Mock
13. ADA
14. HXB2
15. YU-2
16. MACS1-BR
17. UK1-BR
18. NL4-3Int-
SVG
C
1st round
2nd round
3rd round
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
1. Molecular weight markers
2. No template
3. SVG genomic DNA
4. 293T genomic DNA
negative controls
5. PM1 cells infected with HIV-1 89.6
6. MDM infected with HIV-1 ADA
positive controls
7. Macrophages
8. Astrocytes
9. Neurons
patient 1 (HIV+)
10. Macrophages
11. Astrocytes
12. Neurons
patient 2 (HIV+)
13. Astrocytes
patient 3 (HIV-)
14. Macrophages
15. Astrocytes
patient 4 (HIV-)
16. Astrocytes
patient 5 (HIV+)
17. Macrophages
18. Astrocytes
patient 6 (HIV+)
19. Astrocytes
patient 7 (HIV+)

Figure 2

but the physiological relevance of this is questionable. In fact, the possibility that infected astrocytes may lack integrated HIV-1 is supported by studies demonstrating high levels of unintegrated HIV-1 DNA in homogenized brain tissue (Pang *et al*, 1990), paucity of viral antigen production by HIV-1-infected astrocytes *in vivo* (Gonzalez-Scarano and Martin-Garcia, 2005), and an integration-independent pathway of HIV-1 in astrocytoma cells (Clarke *et al*, 2006).

Although non-productive astrocyte infection by HIV-1 *in vivo* is a well-established consequence of HIV-1 infection of the CNS, whether HIV-1 integrates in astrocytes *in vivo* remains an important but unresolved question. The major obstacles that have prevented this question from being addressed include the lack of reliable methods to isolate pure cell populations from well-characterized tissues, the difficulty of ensuring cell purity, and the complexity and sensitivity of the molecular techniques required. In this study, we sought to determine whether astrocytes in autopsy brain of HIV-1-infected subjects contain integrated HIV-1 DNA by combining double immunohistochemical staining and laser capture microdissection to identify and isolate specific cell populations, genetic analysis of HIV-1 sequences in the microdissected cell populations to confirm cell purity, and triple-nested *Alu*-PCR to detect integrated forms of HIV-1 DNA.

Autopsy brains of 5 subjects who died with HIV-associated dementia (HIVD) and 2 HIV-1-negative age- and sex-matched control cases were studied in accordance with guidelines endorsed by the Alfred Hospital and Johns Hopkins University human ethics committees. The severity of dementia was quantified utilizing the Memorial Sloan-Kettering (MSK) criteria at both of the clinical sites (Price and Brew, 1998). Neuropathological examination of tissue sections derived from frontal lobe demonstrated white matter microglial nodules associated with multinucleated giant cells in 3/5 HIVD cases, or sporadic foci of microglial accumulation in 2/5 HIVD cases, reflecting differences in the pathological severity of HIV-1 encephalitis (HIVE). The identification of astrocytes, macrophages, and HIV-1 antigen-positive cells was achieved using the EnVision Plus double immunohistochemical (IHC) staining system (DakoCytomation, Denmark), and cell-specific antibodies that have been described previously (Thompson *et al*, 2004). HIV-1 protein was confirmed in macrophage-lineage cells by HIV-1 p24 and CD68 staining, and astrocytes were identified and distinguished from macrophage-lineage cells by glial fibrillary acidic protein (GFAP)/CD68 staining (Figure 1A). Neurons were identified in hematoxylin and eosin stained sections by recognition of classical features including central nuclei with central nucleoli, oriented apical dendrites, and variably granular cytoplasm (data not shown).

After ablating the cytoplasm and surrounding matter to eliminate possible contamination by adjacent cells, 100 nuclei of each cell type were isolated from 6 μm formalin-fixed, paraffin-embedded tissue sections using a PALM® laser capture microscope (PALM Microlaser Technologies, Germany) (Figure 1B,C) and genomic DNA extracted. DNA integrity was verified by amplification of GAPDH DNA (Figure 2C), as described previously (Thompson *et al*, 2004). The purity of the microdissected cell populations was initially assessed by microscopic examination of sections before and after microdissection (Figure 1B,C), and confirmed genetically by phylogenetic segregation of HIV-1 V3 envelope sequences between astrocyte and macrophage populations [(Thompson *et al*, 2004), and data not shown].

An *Alu*-PCR protocol (Brussel and Sonigo, 2003) was modified to detect integrated HIV-1 DNA in astrocytes (Figure 2A). The R-U5 region of the HIV-1 long terminal repeat (LTR) was amplified from integrated HIV-1 provirus using *Taq* DNA polymerase and triple nested *Alu*-PCR. The initial PCR used primers Alu 1 (5′-TCCCAGCTACTGGGGAGGCTGAGG-3′), Alu 2 (5′-GCCTCCCAAAGTGCTGGGATTACAG-3′) and M13RLTRAlu (5′- GGATAACAATTTCACACAGGCT GGCTAACTAGGGAACCCACTG-3′) to amplify HIV-1- and *Alu* repeat-containing fragments of varying length from genomic DNA. The M13RLTRAlu primer

Figure 2 Detection of integrated HIV-1 in astrocytes. (**A**) Schematic of the modified *Alu*-PCR protocol. The modified *Alu*-PCR has design features that ensure amplification of only integrated forms of HIV-1 DNA, including initial 3′ and 5′ primers targeted to genomic *Alu* repeat sequences and a 5′ primer targeted to the R region of the HIV-1 LTR that also introduces a non-HIV-1 M13 sequence, and secondary 5′ primers targeted to just the M13 sequence incorporated into the first round amplimers. Since only a small fraction (2–3%) of the microdissected astrocytes are likely to contain HIV-1 DNA (Brack-Werner, 1999), a third primer set that targets the R-U5 region of the HIV-1 LTR was used to increase the sensitivity of the PCR. (**B**) Detection of integrated HIV-1 DNA in SVG astrocytes and Cf2th-CD4/CCR5/CXCR4 cells 72 hours after infection with equivalent amounts of laboratory adapted (NL4-3), primary blood-derived (ADA), and primary brain-derived (YU-2, UK1-br, MACS1-br) (Gorry *et al*, 2001) strains of HIV-1. Negative controls included genomic DNA obtained from uninfected SVG cells, and DNA obtained from Cf2th-CD4/CCR5/CXCR4 cells and SVG cells inoculated with NL4-3Int-that has a stop codon in the integrase gene. Positive controls included DNA extracted from PM1 cells infected with HIV-1 89.6. DNA integrity was verified by amplification of GAPDH DNA. The data shown are representative of 3 independent experiments. (**C**) Detection of integrated HIV-1 DNA in nuclei of astrocytes and macrophage-lineage cells, but not neurons, which were isolated from autopsy brain tissue sections by laser capture microdissection. Positive controls included DNA extracted from PM1 cells and MDM infected with the 89.6 or ADA strains of HIV-1, respectively. Negative controls included genomic DNA obtained from uninfected SVG and 293T cells, neurons isolated from brain of 2 HIV-1-infected subjects (patients 1 and 2), and macrophages and/or astrocytes isolated from brain of 2 HIV-1-negative controls (patients 3 and 4). DNA integrity was verified by amplification of GAPDH DNA. The data shown are representative of 2 independent experiments.

used in the first round PCR also contains M13 DNA sequences that serve as a template for subsequent second round priming. This is a specific design feature that ensures that the second round PCR amplifies only products generated in the first round PCR, and not additional unintegrated forms of HIV-1 DNA. The initial, first round PCR reaction consisted of an initial denaturation step of 95°C for 2 min, then 12 cycles of 95°C for 15 sec, 57°C for 15 sec, and 72°C for 170 sec, followed by a final extension of 72°C for 7 min. The second round PCR used primers M13Int (5′-GGATAACAATTTCACACAGG-3′), which is directed against the M13 DNA sequence generated in the first round PCR amplimers, and U5Int (5′-CTAGAGATTTTCCACACTGAC-3′) that corresponds to nucleotide positions 9687 to 9707 of HIV-1 NL4-3. The second round PCR consisted of an initial denaturation step of 95°C for 2 min, then 35 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec, followed by a final extension of 72°C for 7 min. The third round PCR used primers LTRAlu1 (5′-CTGGCTAACTAGGGAACCCACTG-3′) and nLTR3′#2 (5′-AAAAGGGTCTGAGGATCTCT-3′) that correspond to nucleotide positions 9568 to 9591 and positions 9665 to 9685 of HIV-1 NL4-3, respectively. The third round PCR consisted of an initial denaturation step of 95°C for 2 min, then 35 cycles of 95°C for 15 sec, 53°C for 15 sec, and 72°C for 30 sec, followed by a final extension of 72°C for 7 min. PCR products were visualized by electrophoresis in 1% (wt/vol) agarose gels and ethidium bromide staining. The triple nested approach with a total of 82 cycles was determined to be optimal for the detection of integrated HIV-1 DNA in microdissected astrocytes and macrophages (data not shown). The assay has the sensitivity to detect integrated HIV-1 DNA in 2 formalin-fixed cells, which was determined by infecting monocyte-derived macrophages (MDM) with HIV-1 ADA and generating DNA standards from nuclei of 100 CD68+/p24+ MDM that were microdissected from formalin-fixed cytospins (data not shown). The primers used in the third round can potentially amplify unintegrated HIV-1 DNA. However, when used alone these primers lack the sensitivity to amplify detectable HIV-1 DNA from microdissected nuclei (data not shown), suggesting that amplimers generated by these primers in the current study are from template DNA generated in the first and second rounds of PCR.

The *Alu*-PCR method was validated by detecting integrated HIV-1 in cultured SVG astrocytes (Major *et al*, 1985) that were infected with different laboratory adapted and primary HIV-1 strains including brain-derived viruses (Gorry *et al*, 2001) (Figure 2B). Cf2th-CD4/CCR5/CXCR4 cells that are fully permissive to HIV-1 infection (Gorry *et al*, 2001; Gray *et al*, 2006) were infected in parallel and used as positive controls. Proviral DNA was detected in SVG and Cf2th-CD4/CCR5/CXCR4 cells infected with laboratory adapted or primary HIV-1 strains, but not in cells infected with an HIV-1 integrase mutant. Similar results were obtained with U251MG astrocytoma cells (data not shown). HIV-1 DNA was not detected in parental Cf2th cells that lack HIV-1 receptors when inoculated with the same viruses (data not shown). The lack of detectable HIV-1 DNA in cells infected with the integrase mutant and parental Cf2th cells provides quality assurance data that contamination was not the source of amplified HIV-1 DNA. These data demonstrate the specificity of the *Alu*-PCR for detecting integrated HIV-1, and its utility for detecting proviral DNA in astrocytes.

Integrated HIV-1 was detected in macrophages and/or astrocytes isolated from brain of 5 HIV+ cases, but not in neurons isolated from 2 of the HIV+ cases (Figure 2C). Sequence analysis showed that the amplimers were HIV-1 DNA sequences that were distinct from common laboratory strains (data not shown). HIV-1 DNA was not detected in macrophages and astrocytes isolated from brain of 2 HIV− controls. The lack of detectable HIV-1 DNA in neurons from HIV+ cases, and in the different cells from HIV− control cases attests to cell purity and lack of contamination in the PCR. Our results demonstrate that HIV-1-infected astrocytes and macrophage-lineage cells in brain contain integrated HIV-1 DNA. However, since the *Alu*-PCR is not quantitative, we were unable to draw an association between the pathological severity of HIVE in the study subjects and level of HIV-1 integration in brain macrophages and astrocytes. Furthermore, because the *Alu*-PCR amplifies HIV-1 sequences that are located adjacent to multiple *Alu* repeat sequences (Figure 2A), we were unable to identify specific genomic sites of HIV-1 integration. Further studies are required to determine the level and location of HIV-1 integration in the individual brain cell types.

HIV-1 cannot be eradicated by antiretroviral therapy alone (Siliciano and Siliciano, 2004). The major obstacle to eradicating HIV-1 by currently available antiretrovirals is the ability of proviral DNA to persist latently in reservoirs such as resting memory T-cells (Siliciano and Siliciano, 2004). In a resting state these cells are an effective sanctuary for HIV-1, but can generate progeny virions when appropriately stimulated. That virus can be activated from these cells provides some hope that immunological or chemical activation strategies may be developed as novel adjunctive therapies to "flush out" HIV-1 from virus sanctuaries (Jacobson, 2005). Combined with antiretrovirals, adjunctive therapies of this nature may potentially eradicate HIV-1 from reservoirs such as resting memory T-cells.

The demonstration of integrated HIV-1 in astrocytes has important consequences for strategies aimed at eradicating HIV-1. Firstly, there is little evidence of productive HIV-1 infection in astrocytes *in vivo* (Gonzalez-Scarano and Martin-Garcia, 2005) suggesting that virus activation from astrocytes in the

brain is unlikely to occur, even under inflammatory conditions that accompany HIVE. This is supported by *in vitro* studies showing that HIV-1-infected astrocytes are relatively resistant to reactivation of virus under conditions that can readily reactivate HIV-1 from latently infected T-lymphocyte and promonocytic cell lines (Gorry *et al*, 2003). Secondly, astrocytes are terminally differentiated brain cell types that are critical for maintaining brain homeostasis (Volterra and Meldolesi, 2005). Consequently, even if astrocytes could be activated to release HIV-1, targeting them for destruction would cause unacceptable adverse effects.

In addition to being an obstacle for HIV-1 eradication, astrocytes harbouring proviral HIV-1 DNA may also contribute to the development of HIVD by a number of potential mechanisms [reviewed in (Gorry *et al*, 2003)]. In HIVD, it is clear that a number of normal astrocyte functions are disrupted. The blood brain barrier is compromised, with parenchymal entry of serum proteins. There are numerous activated microglia, excessive levels of neurotoxins and evidence of neuronal apoptosis. Using highly sensitive *in situ* PCR detection methods, our previous studies demonstrated a greater number of HIV-1 DNA positive astrocytes in post-mortem brain tissue from individuals with rapidly progressive dementia, compared to samples from non-demented subjects or those with slow progressing dementia (Thompson *et al*, 2001). Astrocyte infection may also lead to astrocyte apoptosis, which is a pathological hallmark of HIVD (Gorry *et al*, 2003). To this end, our previous studies also showed a strong correlation between the degree of astrocyte apoptosis and the presence of HIVD, and also the rate at which the dementia progresses (Thompson*et al*, 2001). Thus, although astrocyte infection with subsequent astrocyte dysfunction and/or death appears to contribute significantly to the development of HIVD, further studies are required to elucidate the pathological mechanisms surrounding the astrocyte reservoir that contribute to HIV-1 neuropathogenesis.

In conclusion, the present study has provided *in vivo* evidence that brain astrocytes, as well as macrophage-lineage cells, are reservoirs of proviral HIV-1 DNA. In addition to the likely deleterious effects that HIV-1 infection will have on astrocyte functions, the findings provide important insights relevant for the development and likely success of strategies aimed at eradicating HIV-1. Specifically, the ability of HIV-1 to integrate in astrocytes *in vivo* suggests that completely eradicating HIV-1 provirus from infected individuals is unlikely to be achievable.

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