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Human immunodeficiency virus type 1 RNA Levels in different regions of human brain: Quantification using real-time reverse transcriptase–polymerase chain reaction

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Human immunodeficiency virus type 1 (HIV-1) enters the central nervous system shortly after the infection and becomes localized in different regions of the brain, leading to various neurological abnormalities including motor disorders and neurocognitive deficits. Although HIV-1–associated functional abnormalities of the central nervous system (CNS) can be evaluated during life by using various test batteries, HIV-1 virus concentration in different brain regions can be measured only after death. The tissues obtained at autopsy provide a valuable source for determining the role of various factors, including that of HIV-1 viral load in the CNS, that may contribute to the regional CNS neuropathogenesis. For this study, we obtained from the National Institutes of Health–sponsored National NeuroAIDS Tissue Consortium (NNTC) the tissues from different brain regions collected at autopsy of HIV-1–positive ($N = 38$) and HIV-negative ($N = 11$) individuals, with postmortem intervals of 2 to 29 h, and measured HIV-1 RNA concentration in the frontal cortex, frontal cortex area 4, frontal cortex area 6, basal ganglia, caudate nucleus, putamen, globus pallidus, substantia nigra, and cerebrospinal fluid. Because HIV-1+ individuals were infected with the virus for up to 21 years and the majority of them had used highly active antiretroviral therapy (HAART), we used highly sensitive real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assay in order to detect a wide dynamic range of HIV-1 RNA with lower detection limit of a single copy. The primers and probes were from the long terminal repeat (LTR) region of HIV genome for achieving higher specificity and sensitivity of detection and amplification. Our results demonstrate a wide variation in the concentration of HIV-1 RNA in different brain regions (5.51 and 8,144,073; $\log_{10}$ 0.74 and 6.91 copies/g tissue), and despite the high specificity and sensitivity of this method, viral RNA was not detected in 50% of all the samples, and in 30% to 64% of samples of each region of HIV-1+ individuals. However, the highest concentration of viral RNA was found in the caudate nucleus and the lowest concentration in the frontal cortex and cerebrospinal fluid. The viral RNA was undetectable in all samples of HIV-negative individuals. *Journal of NeuroVirology* (2007) **13,** 210–224.

Keywords: brain regions; HIV-1 RNA; real-time RT-PCR

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Introduction

Human immunodeficiency virus type 1 (HIV-1) enters the central nervous system (CNS) shortly after infection and gets localized in different brain regions (Resnick *et al*, 1988; Wiley *et al*, 1986). Although, the mode of entry of HIV-1 into the CNS still remains debatable, the proposed models include its neuroinvasion through trafficking of infected monocytes and possibly lymphocytes across the blood-brain barrier (Nottet *et al*, 1996; Persidsky *et al*, 1997), and/or through transfer of the virus via the CNS vascular endothelium (Bagasra *et al*, 1996; Maslin *et al*, 2005). However, productive infection in the CNS is established after reentry of HIV-1 from the peripheral system subsequent to systemic immune-suppression and progression to acquired immunodeficiency syndrome (AIDS), characterized by the loss of CD4+ cells, the main peripheral target of invasion by HIV-1. In the CNS, however, the principal targets of HIV-1 invasion are macrophages and microglia, and the virus uses CD4 receptors, together with the chemokine receptors CCR5 as the predominant coreceptor, for its entry inside the cells (Premack and Schall, 1996; He *et al*, 1997; Gabzuda and Wang, 1999). These infected macrophages and microglia then become a significant cellular reservoir for long-term viral persistence in the CNS (Pierson *et al*, 2000) and play a major role in the development of HIV dementia (HIVD) (Kaul *et al*, 2001). Late in the course of HIV-1 infection and in the absence of opportunistic infections, 30% to 60% of all HIV-1–infected individuals and 25% to 30% of AIDS patients develop a number of neurological disorders, including AIDS dementia complex (ADC), a disorder of progressive impairment of motor and cognitive functions, including decreased memory, difficulty concentrating, psychomotor retardation, headaches, motor deficits, and psychiatric problems (Navia *et al*, 1986; Budka, 1991, McArthur *et al*, 1994; Price, 1996). Furthermore, HIV-1 in the CNS causes encephalitis with a constellation of histopathologic changes, including reactive astrocytes, myelin pallor, microglial nodules, perivascular inflammation, and multinuclear giant cells formation (leukoencephalopathy) (Budka, 1989; Lipton and Gendelman, 1995). Although, since 1996, addition of triple drugs to the highly active antiretroviral therapy (HAART) regime has brought a significant reduction in the peripheral viral burden, these drugs reach the brain in suboptimal levels, and the CNS becomes a sanctuary site for the virus (Lipton, 1997; Pialoux *et al*, 1997; Schrager and D'Souza, 1998), which in turn can be delivered back to the periphery, resulting in perpetuation of the infection (Kerza-Kwiateki and Amini, 1999; Pierson *et al*, 2000). Moreover, the cerebrospinal fluid (CSF) is also implicated as an independent reservoir of viral replication and a possible sanctuary site for the virus, as evidenced by the findings of studies showing high levels of HIV-1 RNA in the CSF of AIDS patients who were neuropsychologically impaired (Ellis *et al*, 1997). Additional complexity is presented by the recent reports suggesting substantial differences in the nucleotide sequence and biological characteristics of HIV-1 derived from the brain and blood of the same patient, suggesting that HIV-1 present in CNS-specific cellular targets may be genetically distinct from those in the periphery (Korber *et al*, 1994; Wong *et al*, 1997). These differences may contribute to the strain of virus resistant to the current antiretroviral therapies (Cunnigham *et al*, 2000; Venturi *et al*, 2000; Smit *et al*, 2004). Earlier studies carried out before the era of HAART found that the persons dying with AIDS had the highest concentration of HIV-1 in the subcortical and frontostriatal circuitaries (Wiley *et al*, 1998) and that there was a considerable neuronal loss in the subcortical regions (Wiley *et al*, 1991; Everall *et al*, 1991). Although, HIV-1 was not detected in majority of the neurons (Masliah *et al*, 1992), high concentration of HIV-1 RNA was found in the subcortical regions and in macrophages and microglia of different brain regions (Wiley *et al*, 1999).

Despite the neurons not being directly infected with the virus, quantitative magnetic resonance imaging (MRI) studies of the brain carried out during the asymptomatic phase of HIV-1 infection reported a selective reduction in the volumes of caudate nucleus and white matter that continued to decrease with the progression of infection (Jernigan *et al*, 1993). A substantial loss was also found in the grey matter of HIV-1–infected patients that correlated with the HIV-1 viral burden and was associated with moderate to severe diagnosis of dementia. The HIV-1 viral load in the earlier studies was determined indirectly by detecting the HIV-1 envelop proteins gp41 and gp120 and the core protein gp24, using techniques with variable sensitivity that included the double-labeling semiquntitative immunohistochemical assays and enzyme-linked immunoabsorbant (ELISA)-based antigen capture assay (Kure *et al*, 1990; Achim *et al*, 1994; Royal *et al*, 1994). Subsequently the quantitative polymerase chain reaction (PCR) assay was used for detecting HIV-1 RNA in body fluids (Mulder *et al*, 1994) and different regions of the postmortem brain tissue of AIDS patients (Wiley *et al*, 1998) by using the Amplicor Monitoring Test (Roche Diagnostic Systems, NJ), which targets the gag region, and the Quantiplex branched-DNA (bDNA) Signal Amplification System (Chiron Corporation Emeryville, CA), which assesses the pol region of the HIV-1 genome (Dewar *et al*, 1994). Although the range of sensitivity of detection by these quantitative techniques were low (400 to 500 HIV-1 RNA copies/ml plasma, a linear range of 400 to 750000 copies/ml), they were found useful for detecting HIV-1 in the human brain regions (Wiley *et al*, 1998; McClernon *et al*, 2001). Although, the sensitivity has improved in the ultrasensitive versions of these methods and now up to 50 to 100 HIV-1 RNA copies/ml plasma can be detected (Sun *et al*, 1998; Collins *et al*, 1997), the quest has continued for

further improving the sensitivity, because HAART is effective only partially to suppress HIV-1 replication, and does not completely eradicate the virus, which continues to reside latently at a low level within the resting CD4+ cells (Chun *et al*, 1997; Siliciano and Siliciano, 2006), and frequently escapes detection due to the limited sensitivity of the above methods. In fact, these methods are mainly used to determine the plasma viral load in order to evaluate the effectiveness of HAART (Persaud *et al*, 2000; Fischer *et al*, 2000). Recently, the introduction of real-time reverse transcriptase–PCR (RT-PCR), also known as the TaqMan 5′ exonuclease assay, has significantly contributed to improve the sensitivity and specificity for detection and quantification of even a single copy of RNA and DNA of various types of viruses present in body fluids, cell cultures, and tissues (Mackay *et al*, 2002). The real-time RT-PCR quantifies the initial amount of the template by utilizing the 5′-3′ *Thermus aquaticus* DNA polymerase and monitors the fluorescence emitted during the reaction and accumulation of the PCR products during each cycle (Holland *et al*, 1991). Additionally, the sample handling steps required by the other PCR methods are circumvented, thus preventing the potential contamination by the carryover PCR products, which frequently contribute to false estimates. In addition to the advantage of its high sensitivity for detecting a single copy, the real-time PCR assay procedure is less labor intensive, much faster with higher throughput, and extremely accurate for measuring HIV-1 RNA and proviral DNA in a number of samples at a time. However, investigations have remained scarce on the use of real-time RT-PCR for measurement HIV-1 virus in the human brain. The purpose of this investigation was to detect and quantify as low as a single HIV-1 RNA copy in different regions of autopsied brains of individuals who had been infected with HIV-1 for various numbers of years and died of AIDS. Moreover, a majority of these individuals had been treated with HAART for controlling the replication of HIV-1. The same brain regions of HIV-negative control individuals were analyzed for comparison. (In this paper, the term HIV-1 RNA [vRNA] concentration is used alternatively with viral load [VL]).

Results

The viral RNA load (vRNA) in regional tissues of autopsied brains and CSF of HIV-1–infected individuals was measured using real-time RT-PCR. Before analysis of the brain tissues for HIV-1 RNA, the reliability of each step involved in the assay method was tested. Efficiency and reproducibility of detection was $100.16\% \pm 0.63\%$, and recovery of HIV-1 plasmid DNA determined before the assays of brain tissue samples was $85.5\% \pm 1.5\%$ (mean ± SD).

In this study we did not determine the proviral DNA concentration. The values in Table 1 are of vRNA concentration in the brain regions of HIV-1+ individuals. All brain regions were found to have a wide variation in vRNA levels, ranging from undetectable (ND) to a maximum of 8144073 ($\log_{10}$ 6.91) copies/g tissue. Of the139 samples from nine brain regions analyzed, HIV-1 RNA was not detected in 50% of the samples, and a wide variation in vRNA levels was observed in each brain region of a majority of the cases (3, 11, 12, 16, 22, 27, 29, 31, 33, 35; Table 1). The values of vRNA were higher in the caudate nucleus of greater number of individuals, compared to that found in the FC and CSF. The scatter plot of regional values ($\log_{10}$ vRNA copies/g tissue) of each individual is given in Figure 1. Despite the high sensitivity of the real-time RT-PCR assay (1.18 copies of the standard HIV-1 III B RNA), viral RNA was undetectable in a large number of samples of each region, such as in FC 64% (16/25), FC-4 61.5% (8/13), FC-6 46.1% (6/13), BG 33.3% (5/15), caudate 57.1% (8/14), putamen 30.7% (4/13), GP 58.3% (7/12), and SN 54.5% (12/22) of the cases, and in CSF 28.6% (4/14) of individuals.

With paired *t* test, a significant difference between the $\log_{10}$ values of vRNA in the caudate nucleus and the other brain regions was observed: caudate versus FC ($P < .001$), putamen ($P < .02$), and BG ($P < .05$). No significant difference was observed in the vRNA load between caudate and SN, FC-4, or FC-6.

Among the 12 samples of CSF, 8 samples had variable concentration of HIV-1 RNA, ranging between 8.4 and 8102 ($\log_{10}$ 0.92 to 3.91) copies/ml, and CSF viral RNA did not relate with the corresponding regional vRNA levels in the CNS tissues, such as in cases 20, 22, 24, 26, 28, and 29. The CSF samples of four cases showed no detectable HIV-1 RNA, and two of these (23 and 36) also had no detectable viral RNA in the CNS tissues. Overall examination of the data obtained in this study indicates that the HIV-1 RNA load was neither influenced by gender nor by ethnic background or age of the individual.

Although, HIV-1+ patients had been infected with the virus for various numbers of years before death (<1 to 21 years, mean ± SD, 8.34 ± 5.00, $N = 38$), neither the low, high, or undetectable concentration of HIV-1 RNA, nor the pattern of its distribution in each region, appeared influenced by the number of years of HIV-1 infection. For instance, case 31, who had been infected for 21 years, had high vRNA in his FC-4, FC-6, BG, and SN, whereas case 34, who had also been infected for 21 years, showed no detectable VL in FC-4, FC-6, and the caudate, and had low vRNA in his putamen and GP. Similar variation is observed in other cases infected for 2 to 17 years. Case 12 had been infected for 7 years, and showed higher vRNA in his FC, caudate, and putamen (copies/g, 34402.9, 4611059, 4366458, respectively; Table 1), but case 15, who had been infected for 10 years, did not have detectable vRNA in his FC, but low level of vRNA was found in his BG (copies/g, 4916.4). Case 17, on the

Table 1 Demographic characteristics and viral RNA load in different brain regions of HIV-1+ (cases 1–38) and HIV− (cases 39–49)

								HIV-1 viral load (RNA copies/g tissue)								
Case	Patient ID	Gender	Ethnicity/race	Age at death	Years of HIV	HAART	PMI (h)	FC	FC-4	FC-6	BG	Caudate	Putamen	Globus	SN	CSF
1	1052	F	HW	48	2	Yes H	5	13.94	—	—	27.742	—	—	—	212.8	40
2	2074	M	HW	37	3	Yes H	6	ND	—	—	ND	—	—	—	63.1	ND
3	6050	M	W	41	5	Yes H	3	698.1	—	—	752	—	—	—	16605.6	1289.4
4	6073	M	W	49	6	Yes H	15	ND	—	—	—	ND	ND	ND	ND	—
5	6007	M	W	34	9	Yes H	5	ND	—	—	—	ND	ND	ND	ND	—
6	1021	F	Am Ind	41	5	Yes H	4	ND	—	—	ND	—	—	—	ND	—
7	2066	M	W	35	5	Yes H	6	8.5	—	—	—	—	—	—	—	—
8	4056	M	W	37	2	Yes H	16	ND	—	—	ND	—	—	—	—	—
9	4013	M	Am Ind	43	6	Yes H	5	ND	—	—	ND	—	—	—	ND	—
10	1013	M	Black	58	9	Yes H	5	32.05	—	—	350.9	—	—	—	—	—
11	2033	F	W	58	3	Yes H	6	499.3	—	—	16.1	—	—	—	—	—
12	6037	M	W	41	7	Yes H	6	34402.9	—	—	—	4611059	4366458	—	—	—
13	6052	M	W	39	4	Yes H	11	ND	—	—	10067.1	—	—	—	—	—
14	6009	M	W	53	5	Yes H	8	ND	—	—	4407.8	—	—	—	—	—
15	6011	M	W	50	10	Yes H	7	ND	—	—	4916.4	—	—	—	—	—
16	4049	M	Am Ind/Alaskan	44	9	Yes H	6	—	29373.248	16145.972	—	47200	2369	1196	4220	—
17	5057	M	W	38	12	Yes H	6	—	ND	ND	—	ND	ND	ND	ND	—
18	D0012KE	M	W	33	9	Yes H	14.5	150.8	—	—	—	—	—	—	—	—
19	D0020LP	F	W	35	10	Yes H	29	ND	—	—	—	—	—	—	ND	—
20	D0027RL	M	W	50	6	Yes H	12.5	ND	—	—	—	—	—	—	—	8.4
21	G0035KG	F	W	43	2	Yes H	6.3	ND	—	—	?	—	—	—	5.51	—
22	G0061AD	M	W	33	17	Yes H	16.17	2937.4	—	—	—	—	—	—	2076753	792
23	G0068BS	M	Black	31	5	Yes H	11	ND	—	—	—	—	—	—	ND	ND
24	G0076EG	M	HW	49	14	Yes H	15	ND	—	—	—	—	—	—	ND	333.2
25	H0007GA	M	Native-Hawaiian	45	8	Yes H	2	ND	—	—	—	—	—	—	ND	—
26	10095	MSM	W	42	6	No H	5	—	ND	ND	—	ND	—	ND	ND	550.2
27	10045	F	HW	31	8	No H	9	—	ND	11.57	—	5324	129.6	4473.3	108.3	—
28	CE124	M	W	55	17	Yes H	24	ND	—	—	ND	—	—	—	ND	3713
29	CE144	M	Black	47	13	Yes H	6	62618.7	—	—	879238.2	—	—	—	1570686	8102
30	CE150	M	Native/Alaskan	42	15	Yes H	7	—	ND	ND	—	ND	22.6	ND	ND	—
31	CA110	M	W	43	21	No H	12	—	5557.1	8144073	281054.6	—	—	—	30209	N/E
32	CB164	M	W	35	<1	Yes H	5	—	526.15	867.2	163.6	—	—	—	491.83	N/E
33	CA236	M	W	34	7	No H	4	—	679.71	93.9	—	1002364	9156.6	269131	—	—
34	CA163	M	W	46	21	Yes H	12	—	ND	ND	—	ND	37.41	233.92	—	—
35	CE116	M	W	46	12	Yes H	12	—	177473	203833		16891	3388	—	—	—
36	CC120	M	Black	45	6	No info	8	—	ND	ND	—	ND	312.9	ND	—	ND
37	CC116	M	Asian	32	11	No H	12	—	ND	293.5	—	324.7	260.7	371.13	—	ND
38	CC147	M	Black	50	6	Yes H	15	—	ND	ND	—	ND	ND	ND	—	—
39	3004	M	W	70	N/A	N/A	22	ND	—	—	ND	—	—	—	—	—
40	3006	M	W	79	N/A	N/A	3	ND	—-	—	ND	—	—	—	—	—
41	3007	M	W	38	N/A	N/A	4	ND	ND	ND	ND	ND	ND	—	ND	ND
42	6001	M	W	46	N/A	N/A	6	ND	—	—	ND	—	—	—	—	—
43	3002	M	W	66	N/A	N/A	4	—	ND	ND	—	ND	ND	ND	ND	ND
44	3009	F	W	53	N/A	N/A	5	—	ND	ND	—	ND	ND	ND	ND	ND
45	3013	F	W	64	N/A	N/A	6	—	ND	ND	—	ND	ND	ND	ND	ND
46	3015	F	W	65	N/A	N/A	7	—	ND	ND	—	ND	ND	ND	ND	ND
47	G0180SS	F	Black	51	N/A	N/A	—	ND	—	—	—	—	—	—	ND	ND
48	G0126GB	F	W	51	N/A	N/A	—	ND	—	—	—	—	—	—	—	—
49	Mhbb555	M	Black	35	N/A	N/A	8.5	—	ND	ND	—	ND	—	—	ND	ND

Note. HIV-1 RNA (copies per g tissue) was measured from each brain region and CSF (copies per milliliter). HIV-1 RNA was not detectable (ND) in a large number of samples of each brain region. However, nondetectability of the HIV-1 RNA did not appear to be related to gender, ethnicity, years of HIV infection, use of antiretroviral medication, or to the diagnosis of neurocognitive status (neurocognitive status presented in Table 2).

M = male; F = female; W = white; H = Hispanic; HW = Hispanic white; Am Ind = American Indian; PMI = postmortem interval; H = HAART; ND = not detected; N/A = not applicable; — = not available; N/E = not enough sample. Thirty-three individuals were treated with HAART (yes H), five individuals not treated with HAART (no H) (four, not treated; one, patient 26, had no HAART at death), and for patient 36, information was not available.

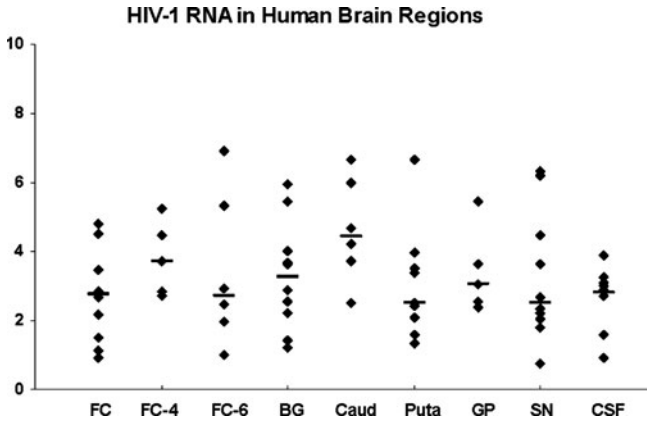

Figure 1 HIV-1 RNA load ($\log_{10}$ copies/g tissue) in each region of HIV-1+ individuals is presented in a scatter plot. The horizontal bars represent the median value in each region. Viral load values were higher in caudate nucleus compared to that in the other regions (FC-4, FC-6, BG, putamen, globus pallidus, SN), and the lowest values were found in FC and CSF.

other hand, has been infected for 12 years but had no detectable vRNA in any of his brain regions.

As for the effect of antiretroviral (ARV) drugs on the regional vRNA load, although the majority of the individuals (33/38) included in this study were taking different combinations of HAART for a variable number of years before death, there was no regulated pattern of regional vRNA found that conformed to the influence of the ARV drugs used by these patients. Moreover, five individuals (cases 26, 29, 31, 33, and 37) who did not use ARV medication also had variable levels of vRNA in different brain regions. For example, among the two individuals (26 and 37) who did not take ARV medication, one patient (26) had no detectable vRNA in any of his brain regions, whereas 37 had undetectable viral RNA in FC-4, but low concentrations were detected in FC-6, caudate, putamen, and GP (copies/g, 293.5, 324.7, 260.7, 371.13, respectively). The other three individuals (29, 31, and 33) had similar range of viral RNA concentration in FC, caudate, putamen and SN, as found in some of the ARV-treated patients (12, 16, 22, and 35).

The vRNA levels also did not appear to be influenced by the postmortem interval (PMI), which ranged between 2 and 29 h (mean $\pm$ SD, 9.41 ± 5.60, $N = 38$), as observed in the values obtained for different tissues in individual cases. For example, case 29 (PMI of 6 h) had high levels of vRNA in FC, BG, SN, as well as in CSF. On the other hand, case 25 (PMI of 2 h) had nondetectable levels of vRNA in FC and SN, whereas case 19 (PMI of 29 h) also had nondetectable viral RNA in FC and SN. The PMI for HIV-negative control cases was 3 to 22 h (mean $\pm$ SD, 7.28 ± 5.44, $N = 9$). The information about the PMI of two HIV− cases was not available. All brain regions of HIV− cases had undetectable vRNA.

The regional VL was very variable in majority of AIDS patients who were diagnosed with probable or possible HIV-associated dementia (HAD) or minor cognitive motor disorder (MCMD). For instance, case 17, who had no detectable VL in any of his brain regions, was also not cognitively impaired, although he had HIV-1 infection for 12 years. On the other hand, case 15 did not have detectable VL in his FC, and had low VL in his BG (copies/g, 4916.4), but he developed possible HAD, with HIV-1 infection lasting for 10 years. Similar findings are observed for other patients.

Discussion

In this study we quantified HIV-1 RNA as a measure of viral load in different brain regions of individuals who had died of HIV-1/AIDS between the years 1998 and 2005, and at autopsy were found to have no significant CNS opportunistic infections (OIs). The same brain regions of HIV-negative individuals who had no psychiatric disorders or OIs, and died during the same span of time (1998 to 2005), were analyzed for comparison (Table 1). The majority of the HIV-1+ individuals (33/38, 87%) had a history of taking ARV drugs when these drugs became available. An increase in life expectancy in these individuals may be attributed to ARV drugs, because these drugs have been documented to dramatically decrease the incidence of mortality and morbidity in different HIV-1–infected populations (Palella *et al*, 1998).

We used the real time RT-PCR and one-step assay, utilizing reverse transcription of the target RNA and PCR amplification of complementary DNA (cDNA) in a single tube using a single buffer system. In order to control for interference by the proviral DNA as a contaminant during the amplification steps of target cDNA, and avoid an error in the estimated values, we treated the tissue lysate with DNase 1 during RNA purification steps. The degraded DNA fragments generated by DNase 1 treatment, as well as small RNA molecules (<200 nucleotides), were eliminated from the silica gel column by repeated washing (described in Materials and Methods). The target HIV-1 RNA contained in the purified total RNA obtained from the brain regions was subjected to reverse transcription and amplification by the TaqMan real time RT-PCR.

The primers and probe selected from the long terminal repeat (LTR) region of the virus genome were used, because these primers and probe with the same base sequence (described in Materials and Methods) have been successfully used earlier and were found to be more specific and sensitive for TaqMan real-time PCR assay of HIV-1 strain III B (Yun *et al*, 2002), compared to those of the pol gene of the virus used earlier with the TaqMan real time RT-PCR assay for determining the viral load in peripheral blood mononuclear cells (Desire *et al*, 2001).

Furthermore, the LTR of the single-stranded HIV-1 RNA genome originates in the unique 5′ (U5), unique 3′ (U3), and the flanking R regions. The multistep process of reverse transcription of HIV-1 RNA results

in the proviral DNA (cDNA) with two identical LTRs, also containing U3 (3′), U5 (5′), and the flanking R regions, placed at either end, that participate in integration of the provirus into the host genome (Levy *et al*, 1993). A number of studies have shown that activity of the LTR regions regulates in part the expression and replication of HIV-1 in the primary host cells including the, CD4+ T cells, monocytes, and macrophages, as well as the monocyte-derived macrophages/microglial cells in the CNS (Tillman *et al*, 1994; Corboy *et al*, 1992). Although, a high HIV-1 DNA viral load in AIDS patients has been reported by a number of studies (Pang *et al*, 1990; Bell *et al*, 1993), most of the DNA in the brain remains unintegrated, because reverse transcription of HIV-1 RNA produces a large amount of nonintegrating type of linear as well as circular cDNA molecules with either one or two copies of the LTR. These nonintegrating type of proviral DNA molecules accumulate in the cell as by-products and do not replicate, but may contribute to the brain viral load (Sonza *et al*, 1994), and have been associated with HIV-1 encephalitis (Pang *et al*, 1990) and HIV dementia (Teo *et al*, 1997). However, because various sites on the LTR regions of HIV-1 genome have been found to be rather hypersensitive to DNase 1 (Verdin *et al*, 1990), we treated the tissue lysates with DNase 1 and eliminated the degraded DNA fragments from the final purified total RNA preparation (described in Materials and Methods) in order to prevent any contribution by proviral DNA molecules during amplification steps. Thus, in this study, quantification of HIV-1 RNA represents the measure of viral load in each brain region.

Moreover, our objective was to utilize the high specificity, sensitivity, reproducibility, and reliability of real-time RT-PCR to detect and quantify a broad dynamic range of the initial template (1 to $1 \times 10^{7-8}$ copies). We were able to detected as low as a single copy of HIV-1 RNA/ng total RNA (5.51 or $\log_{10}$ 0.74 copies/g tissue) in a sample of SN (case 21), and as high as 866 copies/ng total RNA (8144073 or $\log_{10}$ 6.91 copies/g tissue) in a sample of FC-6 (case 29). Because a single copy of RNA is the detection limit of this assay, the undetected HIV-1 RNA in a large number of samples can be reasonably considered to represent the absence of virus in these brain regions. The viral RNA was nondetectable in the CNS regions of all HIV-negative individuals (Table 1).

Additionally, we used the housekeeping genes glyceraldhyde-3-phosphate dehydrogenase (GAPDH) mRNA as well as 18s ribosomal RNA as active references and endogenous controls for indicating RNA integrity, for normalization of the differences in the amount of total RNA added to each reaction, as well as for validation of gene expression of viral RNA. Expression of these genes is unaffected by the experimental conditions used in this study on autopsied brain tissues. To the best of our knowledge, this is the first report to use real-time RT-PCR and the primers and probe from the LTR region of the viral genome for detection and quantification of HIV-1 RNA in the autopsied human brains.

Some of the factors that may potentially influence the estimated CNS regional HIV-1 RNA concentrations were evaluated, i.e., the interindividual variation in PMI, and the possible blood contamination of the tissues with plasma HIV RNA. With regards to PMI of the individual cases included in this study, the autopsy time varied between 2 and 29 h (mean $\pm$ SD, 9.41 ± 5.6 h); in order to minimize the influence of postmortem autolysis, the tissues obtained for this study were within the guidelines of the National NeuroAIDS Tissue Consortium (NNTC) (Morgello *et al*, 2001) and were from the cases autopsied within 24 h of their death. Moreover, a number of studies on the postmortem brains of animals and humans have reported no major effect of PMI of up to 48 h on the concentration or purity of total RNA and on the integrity of mRNA (Barton *et al*, 1993; Johnson, 1986; Perret *et al*, 1988; Bahn *et al*, 2001), as the half-life of mRNA is a few hours even when ribonucleases are active (Hargrove and Schmidt, 1989), and once the mRNA stops being translated, it is less susceptible to ribonuclease digestion. Moreover, the ribonuclease activity is inhibited by the endogenous inhibitors that persist for a longer time after death (Barton *et al*, 1993). There is also a decrease in the availability of adenosine triphosphate (ATP), which provides energy required for catabolism and RNA becomes more stable with the possible inactivation of its normal degradation processes (Morrison and Griffin, 1981). Because real-time PCR quantifies mRNA present in the tissues collected and snap frozen at the time of autopsy, it does not reflect the levels of post-translational changes taking place in the cells. Because a PMI of 9.41 ± 5.60 h was within the guidelines for the cases used in this study, we do not anticipate a significant effect of variable PMI either on the integrity or concentration of mRNA, and therefore on the results of CNS regional viral load in these brain samples.

In order to minimize any potential contribution by the HIV-1 RNA in blood that may be adhering to the CNS tissues, the tissues were rinsed with buffer to remove blood as described in Materials and Methods, and in earlier studies (Achim *et al*, 1994; Wiley *et al*, 1998; McClernon *et al*, 2001). Additionally, to prevent potential error in technical procedures and to keep the variation to a minimum, RNA was extracted from all the tissues at the same time by the same person using the same reagents, protocol, and procedures.

We found that the concentration of HIV-1 RNA was not distributed uniformaly in all the brain regions investigated and there was a wide variation in intra- and interindividual regional values of HIV-1 RNA (Table 1). Moreover, the vRNA was undetectable in 50% of the total samples of CNS tissues assayed, as well as in a large number of samples (30.7% to 64%) of each region. There are only a few studies on the

measurement of CNS regional HIV-1 RNA, and these studies have used the conventional methods to measure the brain HIV-1 RNA concentrations. However, despite the differences in the sensitivity of detection between the real-time RT-PCR assay used in this study (detection limit, a single copy of HIV-1 RNA) and the conventional methods, such as Quantiplex bDNA Signal Amplification System, Roche Amplicor HIV-1 Monitor Test, version 1.0, and the Nucleic Acid Sequence Based Amplification [NASBA], used in the earlier studies (detection limit, $4–5 \times 10^{2-4}$ copies/ml plasma), our findings are consistent with those reported earlier regarding an unequal distribution of HIV-1 RNA in different regions of autopsied human brains (Wiley *et al*, 1998, 1999). Moreover, the pattern of CNS regional involvement for HIV-1 RNA is also comparable with higher VL in some regions, such as caudate nucleus, hippocampus, basal ganglia, and globus pallidus, and low VL in the mid frontal cortex and other regions such as cerebellum. We did not include hippocampus and cerebellum in this study but found higher concentrations of viral RNA in a larger number of samples of caudate nucleus, FC-4, FC-6, and substantia nigra (mean $\pm$ SD, $\log_{10}$ copies/g tissue, 4.63 ± 1.38, 3.80 ± 0.96; 3.55 ± 1.89; and 3.44 ± 1.77, respectively), compared to those in the frontal cortex and CSF (see Figure 1). Other investigators also found wide regional variation in the brain HIV-1 RNA, as well as high levels of vRNA and HIV antigens in the caudate nucleus (McClernon *et al*, 2001), and a preponderance of productive HIV-1 infection within the basal ganglia, brain stem and deep white matter (Kure *et al*, 1990), and atrophy of the caudate in HIV-associated dementia (Kieburtz *et al*, 1996).

Use of HAART may be considered as one of the contributing factors for the variability in intra- and interindividual CNS regional HIV RNA observed in these individuals, because a majority (70%) of the cases included in this study used specific combination of antiretroviral drugs. However, despite these medications, their neurocognitive deficits persisted, possibly due to the poor CNS penetration of most drugs in HAART. The suboptimal concentrations reaching the brain may also have variable regional distribution and induce the development of drug-resistant virus. Surprisingly, the five individuals (26, 27, 31, 33, 37) who did not have HAART were also long-term (6 to 21 years) survivors of HIV-1 infection and manifested neurocognitive problems similar to those who were treated with HAART, and the regional variation in their HIV-1 RNA concentrations was also similar to those who used HAART. The variable effect of antiretroviral drugs on brain viral load was also reported by a recent study showing that HIV-1–infected individuals who had taken different combination of HAART even 3 months before death had significantly lower viral RNA (measured by the Amplicor Standard Assay, detection limit, 400 copies HIV-1 RNA) in the frontal cortex and other brain regions as compared to those who did not take HAART (Langford *et al*, 2006), and there was no significant difference in the viral RNA between the subjects who took one CNS-penetrating antiretroviral drug (2.0 to 3.5 $\log_{10}$ copies) and those who took two penetrating drugs (1.7 to 4.8 $\log_{10}$ copies), although the viral RNA was slightly higher in the subject who took one penetrating drug. Moreover, this study also found that one subject who continued to take HAART for more than 2 months before death had lower brain viral load (2.8 $\log_{10}$ copies) than a subject who continued to take the drug until 1 day before his death (4.2 $\log_{10}$ copies). These findings also indicate an inconsistency of the effect of HAART on CNS regional HIV-1 RNA concentration in HIV-infected individuals.

The CNS regions included in this study for measurement of viral RNA have been associated with the regulation of various neurocognitive functions, and majority of the HIV-1+ individuals when assessed during life were neurocognitively impaired. The limitations in the study that precluded the delineation of statistical relationship between the regional vRNA levels and neurocognitive status included (1) unavailability of tissue sample of each region from every individual; (2) a wide intra- and interindividual variability found in the regional vRNA concentration; and (3) a large number of samples having undetectable vRNA. However, when we determined the cumulative concentration of vRNA ($\log_{10}$ copies/g tissue) in the CNS of individuals who had diagnosis of probable or possible HAD, probable or possible MCMD, and whether they were impaired or normal (Table 2), the data revealed that low, high, or not detectable vRNA did not correspond to any specific category of neurocognitive status. For example, the individual (CA236) who was diagnosed with normal neurocognitive functions was found to have high vRNA levels (20.199 $\log_{10}$ copies/g tissue), and these levels were similar to that of CE144 (20.844 $\log_{10}$ copies/g tissue) who was diagnosed with MCMD. Patient 6007, who was diagnosed with probable HAD, had nondetectable vRNA levels; however, patient 5057, who was not impaired, also had nondetectable vRNA levels. Nondetectable HIV-1 RNA was also found in some of the individuals who had probable (4007) or possible HAD (6073, 4056) or MCMD (G0068BS).

Similar findings in the relationship between the brain viral load and neurological problems have been reported in different earlier studies. For example, one study detected high concentration of viral RNA in the brains of AIDS cases, but was undetectable in the presymptomatic HIV-1+ intravenous drug users (Bell *et al*, 1993), whereas another study found that neurocognitive impairment was associated with high levels of HIV-1 RNA in the CSF (Ellis *et al*, 1997). Using Nucleic Acid Sequence Based Amplification Assay (NASBA; with lower limit of detection 5000 to 10,000 copies/g tissue), McArthur *et al* (1997) measured HIV RNA in the brain regions (midfrontal gyrus

Table 2 Neurocognitive status and cumulative concentration of HIV-1 RNA (vRNA, $\log_{10}$ copies/g tissue) in CNS of each HIV-1+ Individual

Probable HAD	vRNA	Possible HAD	vRNA	Probable MCMD	vRNA	Possible MCMD	vRNA	MCMD	vRNA	Impaired	vRNA	Not Impaired	vRNA	Normal	vRNA
1052	6.51	6073	ND	2074	1.80	D0027RL	0.924	CE144	20.844	G0035KG	0.74	5057	ND	CA236	20.199
6050	11.04	4056	ND	G0068BS	ND	CE116	18.314	CC147	ND	G0076EG	2.522	CC116 no DX	18.314	CA163	3.94
6007	ND	6052	4.00	H0007GA	ND				CE124	3.569					
2066	0.91	6011	3.69	10095	2.74					CB164	10.56				
4013	ND	4049	23.41	CE150	1.35					1021	ND				
1013	4.055	G0061AD	9.215												
2033	3.91	10045	12.585												
6037	17.836	CA110	20.58												
6009	3.64														
D0012KE	2.178														
D0020LP	ND														
CC120	2.495														
N = 12		N = 8		N = 5		N = 2		N = 2		N = 5		N = 2		N = 2	

and basal ganglion) and the CSF of demented and nondemented subjects and found a wide range of HIV RNA levels, from undetectable to 8.14 log copies/g tissue, and surprisingly, found no difference between HIV RNA concentrations in the midfrontal gyrus of some of the demented and nondemented cases (mean log copies/g tissue, 4.55 ± 0.44 and 4.56 ± 0.40, respectively). Moreover, although the overall HIV RNA levels in the two brain regions (midfrontal gyrus and basal ganglion) were highly intercorrelated ($r = .73$, $P = .038$), levels of HIV RNA in both brain regions of only demented subjects had a weak correlation with those of their antemortem CSF ($r = .33$). Thus, HIV-1 RNA concentration in all regions of human brain may not always be directly associated with HIV dementia.

A few other reports have also described wide variations in the CNS regional HIV RNA levels as well as their relationship with neurocognitive deficits in nondemented and demented HIV-infected individuals. McClernon *et al* (2001) used the NASBA QT assay and found that the CNS regional HIV RNA levels were widely variable within each group of nondemented, mildly as well as moderately demented patients, and although there was a significant difference in the CNS viral RNA between nondemented and moderately demented ($P = .0002$) and between mildly and moderately demented ($P = .0128$) subjects, interestingly, some of the nondemented HIV-1–infected individuals were found to have higher viral load than the demented patients. Moreover, treatment with zidovudine (AZT), although resulted in a decrease in the brain VL of some of the patients, also caused one or two reverse transcriptase mutations (McClernon *et al*, 2001).

In summary, we measured HIV-1 RNA in different regions of the brains of individuals who were infected with HIV-1 for various numbers of years and died of HIV/AIDS. The majority of these individuals had received HAART for various numbers of years, but despite HAART their neurocognitive deficits persisted. Because HAART treatment decreases the circulating viral load to below the limit of detection by most conventional assays, with detection limit of 4×10^2 copies/ml, and considering the unpredictable effect of HAART on brain viral load, we used highly sensitive real-time RT-PCR for detecting a wide dynamic range of HIV-1 RNA in different CNS regions, from a single copy to $1 \times 10^{7-8}$ copies/g tissue. We found a wide variation in the intra- and interindividual CNS regional HIV-1 RNA levels (copies/g tissue), irrespective of age, gender, ethnic background, the number of years of infection, neurocognitive status, or whether during life the individuals had received HAART intervention or not.

Materials and methods

Postmortem human brain tissues

Postmortem human brain tissues from different regions of HIV-1–seropositive and HIV-negative control individuals used in this study were procured from the four centers of the NIH-supported National NeuroAIDS Tissue Consortium (NNTC). Each of these centers obtained the informed consent of the families, and the approval of their Institutional Review Board for human ethics, for enrollment, evaluation, and follow up of these patients every 6 months during life, collecting data on various aspects of their health, including physical, physiological, mental, cognitive, and neuropsychological functions; for autopsy; and for donation of organ and body fluids as gifts after their death (Anatomical Gift Act, 1990). At the NNTC Centers, HIV-1–seropositive status of individuals is evaluated at the time of enrollment and at 6-monthly intervals before death; at autopsy, the brain and other body tissues are harvested for research investigations related with HIV/AIDS. The NNTC guidelines for postmortem interval (PMI) are within 24 h at all centers. All tissues after harvesting are immediately transferred to separate sterile containers, appropriately labeled for specific tissues, and either snap frozen in liquid nitrogen and transferred to -80°C freezers for storage until used, or are fixed in 10% buffered formaldehyde for histopathological studies. Postmortem cerebrospinal fluid (CSF) is collected from the third or lateral ventricles and stored at -80°C (Morgello *et al*, 2001). For this study we used snap-frozen tissues.

This study was approved by the Institutional Review Board of the University of Miami. The tissues obtained for this study were shipped in dry ice according to the guidelines for overnight shipping and handling established by the NIH/NNTC (US Federal Regulations 49 CFR 172 subpart H). The brain tissues of 49 individuals included 38 HIV-1 positive and 11 HIV-1 negative, belonging to different ethnic categories, having no evidence of active opportunistic infection of the CNS at the time of death. The case numbers as well as their demographic characteristics recorded at the NNTC Centers are presented in Table 1. Tissues of HIV-1+ cases, cases 1 to 38, were obtained from the four centers including the National Neurological Brain Bank (NNAB; Los Angeles, CA), Texas Repository for AIDS Neuropathogenesis Research (TRANR; Galveston, Tex), the Manhattan Brain Bank (MHBB; New York, NY), and HIV Neurobehavioral Research Center (HNRC) brain bank at the University of California at San Diego. Tissues of HIV-1–negative cases, cases 39 to 49, were obtained from NNAB, Texas Repository, and MHBB. The age of HIV-1+ and HIV-1− cases were between 31 and 58 years and 35 and 51 years, respectively, with the exception of two HIV-negative individuals (39 and 40), who were of age 70 and 79 years, respectively. Among the 38 HIV-1+ cases investigated, 32 (84%) were male and 6 (16%) were female; 24 (63%) White, 5 (13.3%) African American (AA), 4 (10.5%) Hispanic White, 4 (10.5%) Native American, and 1 (2.7%) Asian. Among the 11 HIV-negative cases, 6 individuals (54.5%) were male and 5 (45.4%) were

female; 9 (81.8%) of them were White, 1 (9.1%) was AA, and 1 (9.1%) was Hispanic Black. The assessment for neurocognitive status of HIV-1+ cases carried out between 1 and 19 months before their death indicated that 12 individuals (31.6%) had probable HIV-associated dementia (HAD), 8 (21%) had possible HAD, 5 cases (13.2) had probable minor cognitive motor disorders (MCMDs), 2 (5.3%) of them had possible MCMD, 2 (5.3%) had MCMD, 5 (13.2%) were cognitively impaired, 1 (2.6%) had no impairment (NPI), 1 (2.6%) had no diagnosis, and 2 (5.3%) were cognitively normal. Individuals excluded from the study were those with neuropsychiatric illness, such as schizophrenia, or opportunistic infection of the CNS; those who had used antipsychotic medication for long term; and those who had history of stimulant drug dependency, CNS cancer, and CNS complications due to severe head injury with loss of consciousness for more than 30 min. The HIV-1+ individuals had died between 1998 and 2005. All HIV-negative individuals were cognitively normal except one (44), who had some impairment not related to HIV or any other infection. The HIV-negative cases included in this study also died during the same span of time (between 1998 and 2005) but due to different causes. The PMI of all cases ranged between 2 and 24 h, except one case (19), who had a PMI of 29 h.

Majority of HIV-1+ cases had used an antiretroviral therapy (ART) regimen prescribed as appropriate for each individual and included the combination of protease inhibitors, single or multiple nucleoside reverse transcriptase inhibitors (NRTIs), plus one nonnucleoside reverse transcriptase inhibitor (NNRTI). Among the five individuals who did not use antiretroviral medication, one (26) had no medication before death, four did not use ART (27, 31, 33, 37), and for one person (36), information was not available.

The brain tissues of different regions of each individual used in this study were characterized and labeled by the respective centers at the time of autopsy and included the frontal cortex (FC), frontal cortex areas 4 (FC-4) and 6 (FC-6), caudate nucleus (Caud), putamen (Puta), globus pallidus (GP), substantia nigra (SN), and cerebrospinal fluid (CSF). Because not all brain regions of each autopsied case were available, we investigated frontal cortex (FC) from 25 HIV-1+ cases and areas FC-4 and FC-6 each from 13 cases. Basal ganglia of HIV-1+ cases ($N=15$) were received as one region, and caudate, putamen, and GP were from 14, 13 and 12 cases respectively, SN was from 22 cases, and CSF from 14 cases. The brain tissues of HIV-1− individuals ($N=11$) included similar regions as those of HIV-1+ cases.

HIV-1 RNA extraction and purification

Total HIV-1 RNA from different parts of the brain tissues was extracted and purified using the RNeasy Lipid Tissue Mini Kit, according to the manufacturer's (QIAGEN) protocol. Briefly, frozen brain tissues (25 to 50 mg) of each region were aseptically and carefully excised, rinsed in buffer (phosphate-buffered saline [PBS], pH 7.4) to remove any possible blood that may be surrounding the tissues to prevent contribution of HIV-1 RNA in blood (although excessive blood drainage at autopsy reduces the volume of blood adhering to the tissues in different brain regions). The tissues were immediately transferred to 2-ml sterile tubes, accurately weighed, and homogenized in 1.0 ml of phenol/guanidine-based QIAzol Lysis Reagent (QLR), designed to lyse fatty tissue such as brain and inhibit RNases. Rotor-Stator homogenizer and a sterile disposable probe was used for disruption and homogenization of the tissues. (Rotor-Stator procedure for tissue disruption and homogenization in the presence of QLR employs the combination of turbulence and mechanical shearing caused by the high speed of the rotor, which thoroughly disrupts and simultaneously homogenizes all the tissue and cells to a liquid within 20 to 40 s.) In order to minimize foaming and avoid denaturation, the tip of the homogenizer was kept submerged in the liquid solution. All the tissue and cells exposed to this procedure of homogenization were simultaneously sheared into small fragments, and all cellular components including high-molecular-weight genomic DNA as well as HIV-1 proviral DNA present in the infected macrophages and microglia would be released into the homogeneous liquid. The lysis mixture of each tissue was then processed for RNA extraction and purification using RNeasy silica gel membrane using the procedure provided with the kit.

Although RNeasy silica gel membrane efficiently removes most of the DNA without DNase treatment, TaqMan real-time PCR is very sensitive to the presence of even a very small contamination of residual DNA, which can interfere in the amplification step and introduce an error in the final values of target RNA. In order to obtain purified RNA (free of DNA) and prevent interference of DNA during amplification, we used RNase-free DNase set (DNase 1) for on-column digestion of DNA, and the degradation products of DNA were eliminated by repeated washings of the RNeasy Mini Spin silica gel column. The washing procedure of the silica gel column also eliminates small fragments (<200 nucleotides) of RNA (5.8s, rRNA, tRNAs) while retaining larger RNA segment (>200 nucleotides). Purified RNA was eluted in a total volume of 60 μl of Rnase-free water, and its concentration was determined spectrophotometrically (Eppendorf BioPhotometer, Model 6131) at absorbance of 260 nm (A_{260}) using RNase-free cuvettes (treated with 0.1 M NaOH and 1 mM EDTA, followed by washing with Rnase-free water). One unit of absorbance at 260 nm corresponded to 40 μg total RNA per ml. Because A_{260} cannot distinguish between RNA and DNA, the ratio of absorbance measured at A_{260}/A_{280} can be used to determine the purity of RNA (the ratio for pure RNA is 1.9 to 2.1). We determined the A_{260}/A_{280} ratio by diluting the RNA solution in 10 mM Tris · HCl, pH 7.5, and the ratio of

1.9 to 2.0 was obtained for all samples that confirmed the purity of RNA preparations without any contamination of DNA. Aliquots of purified total RNA were transferred to siliconized tubes and stored at −80°C until assayed.

Primers and probes for TaqMan real-time PCR

Primers and probes used for the TaqMan real-time PCR assay were from the long terminal repeat (LTR) region of 640 bp, with two identical regions located at both ends of the proviral DNA of HIV-1 subtype III B. The sequences of the sense and antisense primers were 5′-GCC TCA ATA AAG CTT GCC TTG A-3′ (base sequence 522 to 543) and 5′-GGG CGC CAC TGC TAG AGA-3′ (base sequence 626 to 643), respectively, of the LTR. The sequence of the TaqMan probe was 5′-CCA GAG TCA CAC AAC AGA CGG GCA CA-3′ (base sequence 559 to 584). The 5′ end of the probe was labeled with a reporter dye, 6-carboxyfluorescein (FAM) and 3′ end labeled with a quencher dye, 6-carboxytetramethyl rhodamine (TAMRA). These primers and probes have been used earlier for quantification of proviral DNA of HIV-1 IIIB extracted from peripheral blood mononeuclear cells (PBMCs) (Yun *et al*, 2002) and were found to have higher sensitivity (lower C_T value) and specificity compared to that obtained with the sequence of pol region of HIV-1 subtype B genome (Desire *et al*, 2001). Prior to quantification of HIV-1 RNA in the brain tissue, the primers and probes were tested for HIV-1 RNA extracted from plasma of infected patients. The detection efficiency with the real-time RT-PCR was 100.16% ± 0.63% (mean ± SD).

HIV-1 RNA standard and standard curve

The standard for HIV-1 RNA consisted of the sucrose gradient purified HIV-1 strain III B virus and was obtained commercially (Advanced Biotechnologies, Columbia, Maryland, USA), with the particle count of 4.72×10^{10}/ml or $4.72 \times 10^{7}/\mu$l in buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5). The RNA extraction of this standard virus with known number of copies/μl was performed using the Qiamp Mini Elute Virus Spin Kit (QIAGEN, Valencia, CA). The extracted RNA standard stock solution was used for 10-fold serial dilutions to obtain eight working RNA standards of concentrations ranging from $2.36 \times 10^{6}/\mu$l to 0.236/μl or 1.18×10^{7} copies/5 μl to 1.18 copies/5 μl, and used in 25-μl reaction mixture for real-time RT-PCR amplification to generate a standard curve. These concentrations of the HIV-1 RNA standard were selected to be equivalent to the range of values of the virus expected in different brain regions and CSF. The sensitivity of detection was 1.18 copies per reaction. At 100% efficiency of amplification, the slope of the standard curve of the HIV-1 III B RNA was −3.32726 and $R^2 = .996513$.

Measurement of HIV-1 viral load in the brain tissue: real-time quantitative RT-PCR

HIV-1 viral load measurement in the tissues from each brain region as well as CSF was carried out using One-Step reverse transcriptase (RT) and real-time PCR in a single buffer system. The protocol was followed as described by the vendor (Applied Biosystems) for the TaqMan One-Step RT and PCR Master Mix Reagents Kit. However, prior to quantification of HIV-1 RNA concentration in the brain tissue, we performed standardization of various steps, including the recovery, reproducibility, efficiency of detection as well as intra- and interassay percent coefficient of variance (% CV) as influenced by the procedures of extraction, reverse transcription of RNA, as well as HIV-1 plasmid DNA amplification by the real-time PCR.

The TaqMan RT-PCR reaction was performed on 7000 ABI Prism Sequence Detection System (Applied Biosystems, Foster City, CA, USA), which contains a laser-assisted fluorometer to screen fluorescence every 7 s in every well on a 96-well plate. For reverse transcription and PCR of HIV-1 target RNA, One-Step RT-PCR Master Mix without uracil *N*-glycosylase (UNG) was used, in order to prevent removal of uracil bases during reaction (because UNG is active at the same temperature as required for RT reaction). The reaction was carried out in 25 μl volume, and to each TaqMan mixture in the tubes was added 12.5 μl TaqMan One-Step RT-PCR Master Mix, 0.625 μl of 40× Multiscribe and RNase Inhibitor Mix, 1.5 μl (600 nM) of sense primer, 1.5 μl (600 nM) of antisense primer, and 0.2 μl (200 nM) of TaqMan probe with the fluorescent reporter dye, 6-FAM attached to the 5′-end, and a quencher fluorochrome, 6-TAMRA, attached to the 3′-end (Livak *et al*, 1995). Reaction tubes were subjected to the thermal cycling conditions of 30 min at 48°C for reverse transcription, AmpliTaq Gold Activation hold for 10 min at 95°C, followed by 45 cycles of denaturation and annealing/extending steps for 15 s at 94°C and 1 min at 60°C, respectively. The 10-min hold at 95°C is required for Optimal AmpliTaq Gold DNA Polymerase. Real-time fluorescence measurements were performed and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit as determined between cycles. The C_T value reflects the earliest point in the amplification that can be used for quantification of the template. Efficiency of amplification was determined by running standard curves with diluted stock standard solution. The slope of the curve obtained with RNA of the sample was compared with that of the RNA standard transcript. The efficiencies between two different targets (sample RNA and standard RNA transcripts) can be considered equal if the difference of the slope (Δs) is smaller than 0.1. A PCR reaction that amplifies the target sequence with 100% efficiency (E) will double the amount of products with each cycle (standard curve). In order to avoid false positive

results, two control tubes showing no amplification were included: (1) one tube containing sample template but no enzyme and (2) second tube containing enzyme but no template.

In order to check the quality and recovery of RNA extracted from the brain tissues and the efficiency of each reverse transcription reaction, two housekeeping genes were run concurrently as external controls: (1) a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA fragment was amplified with specific primers using TaqMan GAPDH Control Reagents, and (2) 18S ribosomal RNA (rRNA), using TaqMan Ribosomal RNA Control Reagents.

Assay of GAPDH was carried out according to the instructions provided with the kit (Applied Biosystems). Briefly, GAPDH RNA sample was mixed with 2× Master Mix without UNG, 40× Multiscribe and RNAse inhibitor Mix, a sense primer and an antisense primer, and the TaqMan probe with fluorescent reporter dye, 2,7-dimethoxy-4, 5-dichloro-6-carboxyfluorescein (JOE), attached to the 5′ end and the quencher dye TAMRA attached to the 3′ end.

Similarly, the TaqMan Ribosomal RNA assay was carried out according to the instructions provided with the kit (Applied Biosystems). Briefly, the ribosomal RNA sample was mixed with 2× Master Mix without UNG, 40× Multiscribe and RNase inhibitor mix, a sense primer, an antisense primer, and TaqMan probe with the fluorescent reporter dye, VIC, attached to the 5′ end and quencher dye, TAMRA, attached to the 3′ end. The same Universal Thermal Cycling Parameters mentioned above were used for assays of both housekeeping genes.

Intra- and interassay coefficients of variance (% CV) were determined using RNA extracted from the standard HIV-1 III B strain. Four serial dilutions (1.18×10^7, 1.18×10^6, 1.18×10^5, 1.18×10^4 copies/5 μl) and one plate reaction was used. For determining the intra-assay coefficient of variance, four replicates of each of the above four dilutions of the standard were tested. Values of CV ranged between 0.52% and 0.84%. For the interassay coefficient of variance, five different experiments in quadruplicate were performed on different days using the above concentrations of RNA and one plate reaction per day. The interassay% CV ranged between 1.10% and 1.23%.

Efficiency and reproducibility of DNA detection were carried out with different concentrations of HIV-1 plasmid DNA standard and tested by real-time PCR using GENE Amplimer HIV-1 kit (Applied Biosystems). For determination of recovery and reproducibility of extraction and amplification of DNA, frontal cortex (25 to 50 mg) of HIV-1+ individual was lysed in 200 μl of lysis buffer from the DNeasy Tissue Kit (QIAGEN). Aliquots of the lysate of FC were spiked with either 1000 or 3000 copies of a HIV-1 plasmid DNA at 1000 copies/μl from GENE Amplimer HIV-1 Control Reagents kit, and DNA extracted and purified according to the protocol provided in the kit. At 99.4% to 100.7% efficiency and reproducibility of detection, the percent recovery of each concentration of DNA added to the FC lysate ranged between 84% and 87%. The background efficiency (100.2%) was obtained by spiking 1000 copies/μl of HIV-1 plasmid DNA to the DNA solution extracted from BG of an HIV-1+ individual.

Similarly, recovery of DNA from CSF was also determined by using two aliquots of CSF of an HIV-1+ individual. DNA was extracted using the DNeasy Mini Kit (QIAGEN). One aliquot of CSF extract was used to determine the original baseline value of DNA in CSF whereas the other aliquot of CSF extract was spiked with 2500 copies of HIV-1 plasmid DNA (1000 copies/μl, GENE Amplimer HIV-1 Control Reagents kit). Recovery of Plasmid DNA from CSF was 86.5%.

Viral load was expressed as copies/g wet weight of tissue and was derived from HIV-1 RNA copies/total RNA extracted from known weight of each tissue. RNA from HIV-1 III B purified virus was used as the standard (each concentration/5 μl).

Statistical analysis

Analysis of the data for all standardization procedures, including % efficiency, % recovery, and intra- and interassay coefficients of variance (% CV), was performed by using descriptive statistics and are presented as mean ± SD. Because the intra- and interindividual regional viral load values were found to vary widely, i.e., from nondetectable (ND) to 1×10^7 HIV-1 RNA copies/g tissue, the data in Table 1 are presented as obtained for individual samples. The regional values of HIV-1 RNA/g tissue in each individual were $\log_{10}$ transformed and are presented as a scatter plot. A paired *t* test was used to calculate the significance of difference between the $\log_{10}$ HIV RNA values in the caudate (the highest vRNA) and the other brain regions.

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