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Circulating proviral HIV DNA and HIV-associated dementia

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Abstract

Objective—Individuals continue to develop HIV-1-associated dementia (HAD) despite treatment with highly active antiretroviral therapy (HAART). Monocytes/macrophages (M/MΦ) can harbor proviral DNA that is not eradicated by HAART. To determine if HAD is associated with the level of HIV-1 infection within circulating leukocytes, we quantified HIV-1 DNA copy number in peripheral blood mononuclear cells (PBMC), and in PBMC subsets.

Design—Cross-sectional analysis within the Hawaii Aging with HIV Cohort comparing participants with HAD to those with normal cognition (NC).

Methods—Real-time PCR assays assessing HIV DNA copy number/ 1×10^6 cells were performed on PBMC and subsets.

Results—Individuals with HAD ($n = 27$) had a median (interquartile range) of 9.11 (37.20) HIV DNA per 1×10^6 PBMC compared to 0.49 (0.89) HIV DNA per 1×10^6 PBMC in individuals with NC ($n = 22$). Using a univariate analysis in the subset of individuals with undetectable viral load (HAD, $n = 11$; NC, $n = 13$), the odds of HAD attributable to HIV DNA copy number was 2.76 (1.28–5.94), $P < 0.01$. Preliminary analysis of a small subset of patients ($n = 5$) suggested that the primary source of HIV DNA may be the activated M/MΦ(CD14/CD16) subset.

Conclusions—These findings suggest a potentially important association between circulating provirus and HAD.

Keywords

HIV-1; DNA; dementia; provirus

Introduction

HIV-1-associated dementia (HAD) persists among HIV-1-infected individuals despite highly active antiretroviral therapy (HAART) [1–3]. In the pre-HAART era, approximately 15% of patients with low CD4 cell counts progressed to HAD [4]. While the incidence of HAD currently appears to be decreasing, the prevalence has risen from 6.6/100 person years in 1994 to 10.1/100 person years in 2000 [4–6]. A determination of the factors associated with prevalent HAD despite effective suppression of peripheral HIV-1-infection is needed. In the era of HAART, the utility of circulating plasma HIV-1 virus (viral load, VL) measurement as a marker of HAD has decreased [7–10]. We propose that detectable plasma VL is neither necessary nor sufficient for the presence of HAD, and that other factors are involved in the pathogenesis of HAD [5,9,11–14].

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Monocytes/macrophages (M/MΦ) are important in the pathogenesis of HAD. Individuals with HAD have higher percentages of circulating activated M/MΦ [14]; these cells are probably important in the neuropathogenesis of HIV-1 possibly by increased virus trafficking into the central nervous system [1,14,15]. As important HIV-1 reservoirs, M/MΦ could harbor proviral DNA [16–18], suggesting a mechanism of increased risk among individuals with undetectable plasma VL [19,20]. In recent experiments, productive HIV-1 infection of macrophages in SCID mice suggest a role in neurological disease [16]. We were therefore interested in assessing the correlation of proviral DNA with HAD by quantitatively assessing HIV-1 DNA copy number (HIV DNA) in PBMC, and further, to determine the association with activated M/MΦ.

Materials and methods

Cohort and clinical data

The participants for this study were selected from the Hawaii Aging with HIV Cohort (HAHC) following informed consent [21]. This longitudinal cohort was established to examine HAD and other neurologic complications in older (≥ 50 years old) compared to younger (20–39 years old) HIV-1-seropositive individuals living in Hawaii. Less than 5% of the participants in the HAHC identify injecting drug use as a risk factor for HIV-1 infection [6,22–24]. Individuals were excluded for the following reasons: (i) a major psychiatric or neurologic disorder; (ii) a history of head injury with loss of consciousness greater than 1 h; (iii) current or past opportunistic infection with brain involvement; (iv) a diagnosed learning disability; or (v) delirium due to medications at the time of examination. Evaluations included the macro-neurological examination, a medical history questionnaire, a medication/adherence history, a DSM-IV-based substance abuse/dependence inventory, immunologic and virologic laboratory tests, and neuropsychological testing. Research-based neurocognitive diagnoses were established for each participant using the American Academy of Neurology 1991 criteria [25]. Full details of the diagnostic approach within HAHC are provided elsewhere [21,26, 27].

Specimens and assessment of HIV DNA

Specimens were obtained and stored at the time of study entry. Plasma VL (Amplicor HIV-1 Monitor Ultra Sensitive Test, Roche Diagnostics, Switzerland) and CD4 cell counts were performed by a certified clinical laboratory. Genomic DNA was purified from peripheral blood mononuclear cells (PBMC) using standard phenol–chloroform extraction. Alternatively, in some cases, a direct lysis protocol was used. Both are comparable in adequately assessing HIV DNA [28,29]. The DNA samples were stored at -20°C and assayed in batches. The quantity and quality of the DNA was assessed by ultraviolet spectrophotometry and PCR analysis using HLA $\text{DQ}\alpha$ and/or β -globin primers as described previously [30].

The assay for measuring HIV DNA was modified from a previously described real-time PCR method demonstrating reproducible intra-assay (replicates within the same real-time PCR run) and inter-assay (replicates between four different runs) results with mean coefficients of variation of 1.1% and 1.4%, respectively with an estimated detection limit in the range 1.0–5.0 (± 1.0 –3.4) HIV DNA copies [31,32]. Assays were set up with independent standard curves measuring relative viral DNA copy number and cellular equivalent genomic DNA compared to unknown specimens. DNA for control and standards was extracted from 8E5 cell line (ATCC 8993, NIH AIDS Research and Reference Reagent Program, Rockville, Maryland, USA), a T-lymphoblastoid cell line with a single copy of HIV-1 per cell [33,34]. The 8E5 DNA was used as a standard for both the *c-jun* and HIV-1 primers to compare the relative HIV-1 burden with the identical aliquot of standard DNA with each run of unknown specimens. Additional control DNA was prepared from non-HIV-1 infected PBMC as well as an HIV-1 control panel, provided by the NIH AIDS Research and Reagent Reference Program (RRR) [35]. Primers for

viral genes included two HIV-1 primers that target the *gag* gene of HIV-1 [36]: sense, 5'-ATAATC-CACCTATCCCAGTAGGAGAAAT-3'; and antisense, 5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3', which amplified a 115-bp fragment. For the human genomic gene, the *c-jun* primers amplified a 248-bp piece of DNA from the single copy human *c-jun* gene: sense, 5'-CATGGAGTCCCAGGAGVGGATCA-3'; and anti-sense, 5'-GCAACTGCTGCGTTAGCATGAGT-3' (GenBank Accession number J04111). All sample preparations were performed in a flow hood to minimize any cross-contamination and in a separate area away from the thermocycler. Assays were performed by real-time PCR using Quantitect Sybr Green PCR Kit (Qiagen, Valencia, California, USA).

The HIV-1 PCR control panel was used to test the standards in our assay and to test the detection limit of the assay as described previously [35]. HIV DNA standards from the RRR Program were provided as 1, 20, 50 and 100 copies, which were set up in our assay separately and in the presence of 1×10^6 and 1×10^3 PBMC equivalents. A panel consisting of 5 and 20 copies per 1×10^6 PBMC and negative controls (no template and HIV-1-seronegative PBMC DNA) was tested in triplicate. Means and standard deviations for the control standards were: 0, 5.3 (± 3.2), and 18.3 (± 4.3) HIV DNA copies per 1×10^6 PBMC for the respective controls (0, 5, 20 copies).

Initially, each sample and standard, including positive and negative controls, was set up in triplicate containing: Sybr Green Master Mix, 5 μ l glycerol, 15 pmol each primer, and 0.1 μ g sample DNA. PCR cycling conditions were: 95 °C for 15 min, 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s, followed by a 7-min final extension step at 72 °C. At the conclusion of the PCR, a melt curve analysis was programmed with the following conditions: beginning at 48 °C, the temperature increased half a degree every 10 s for 90 cycles. The initial specimen DNA was diluted in concentrations corresponding to 40 000 cell equivalents/10 μ l with the number of cell equivalents calculated assuming that 6.6 ng of DNA was equivalent to 1×10^3 cells [37]. If a specimen had a sample threshold temperature outside the standard threshold temperatures, the assay was repeated with increasing or decreasing amounts of template until the specimen threshold temperature was within the standard threshold temperatures. Initially, the same amount of template was used for both the virus and genomic primer sets. If the result for the virus primer set was below the lowest standard, then another reaction was set up using additional template, depending on how far outside the standard curve the result was. For lower copies (less than 1 copy number per million cells), the results were extrapolated from the standard curves generated by the real-time PCR program, iCycler IQ Software Program, version 3.0 (BioRad, Hercules, California, USA). Thus each specimen was evaluated and quantified individually with the genomic and virus equivalents independently. Stored supernatants recovered from extracted DNA were assayed for HIV DNA, which were undetectable for HIV DNA using appropriate positive and negative controls.

In the analyses, each unknown was analyzed against the standard curve generated by the iCycler IQ Software Program (BioRad) and calculated as HIV DNA copy number per 1×10^6 cells. The reported value was related to the number of genomic equivalents as defined by simultaneous amplification of genomic *c-jun* sequences, as described previously [31].

CD14/CD16 isolation and HIV DNA analysis

A Monoctye Isolation Kit and CD16-positive selection was used on specific cases with the AutoMacs (Miltenyi Biotec Inc., Auburn, California, USA) cell separation machine harvesting both CD16-positive and CD16-negative cell populations. The initial isolation for CD14 utilized a negative selection, which isolated CD14-positive and CD14-negative pools. The CD14-positive cells were negatively selected and further separated via positive selection for CD16. To confirm the separation technique, an aliquot of each subset from representative cases was analyzed by three-color FACSCaliber flow cytometer (BD Biosciences, San Jose, California,

USA) with monocyte, macrophage, and T-cell antibodies. The monocyte population was confirmed to be >95% CD14. DNA from CD14+/CD16+ and CD14- subsets were analyzed for HIV DNA to quantify proviral DNA in PBMC subsets.

Statistical analysis

Statistical analyses were performed combining older and younger groups into one dataset. All tests of hypotheses used only the participants classified as HAD or as cognitively normal. Participants classified as nonsyndromic neuropsychologically abnormal or as having HIV-associated minor cognitive motor disorder were excluded. We used logistic regression to examine the independent effect of HIV DNA on HAD compared to normal cognition (NC) in a univariate model and then in a multivariate model that controlled for the effects of VL, CD4 cell count, duration of infection, HAART, age, education, and ethnicity [38]. We then repeated these analyses in the subset of individuals without detectable VL. Analyses were conducted using SAS 8.2 (SAS, Cary, North Carolina, USA).

Results

The demographic constitution of the cohort used in this study is summarized in Table 1. The quality and quantity of DNA were adequate from all of the specimens. The distributions of HIV DNA were skewed, as reflected in the standard deviations and differences between mean and median HIV DNA values (Fig. 1, Table 2).

Individuals with HAD had a median [interquartile range (IQR)] of 9.11 (37.20) HIV DNA per 1×10^6 PBMC compared to 0.49 (0.89) HIV DNA per 1×10^6 PBMC in individuals with NC (Table 2). In an unadjusted logistic regression model, the association of HIV DNA with HAD resulted in an odds ratio (OR) of 2.83 (IQR, 1.57–5.08; $P < 0.001$). The analyses were repeated after controlling for VL, CD4 cell count, HAART (71% of the participants were on HAART), duration of infection, education, age, and ethnicity which continued to show significance ($P < 0.01$). To evaluate the possibility that these findings were driven primarily by a few participants with very high HIV DNA values, we excluded the participants with the five highest values. This did not substantially change our results. We repeated the univariate analysis in the subset of individuals who had undetectable VL (HAD, 11; NC, 13) to determine whether the effect we observed was attributable to plasma VL. This also did not substantially change the results (OR, 2.76; IQR, 1.28–5.94; $P < 0.01$) (Table 2 and Fig. 1).

Of particular interest were two individuals with HAD who had relatively high HIV DNA and undetectable VL: 114 and 618 HIV DNA per 1×10^6 PBMC. In an attempt to understand potential differences in these two individuals, analyses were performed on these two, and three other specimens, to ascertain HIV DNA in subsets of PBMC. PBMC from the five individuals (three with HAD, including the two with highest HIV DNA per 1×10^6 PBMC, and two individuals with NC) were obtained and separated into CD14/CD16 and CD14-negative subsets. A representative flowcytometer plot is shown in Fig. 2 where 11–15% of the monocytes demonstrate CD14/CD16 phenotype. The CD14+/CD16- subset was not recovered. HIV DNA was measured from each of the retained subsets (Table 3). From the two individuals with relatively high HIV DNA in PBMC, the HIV DNA per 1×10^6 PBMC was the highest in the CD14/CD16 subset (activated M/MΦ) compared to the CD14-negative subset. By flow cytometry, the CD14-negative subset included CD4 T cells. The number of cells lysed from the isolated subsets used to obtain DNA varied depending on the total number of cells originally obtained, ranging from 30 000 to 500 000. Therefore, the HIV DNA per 1×10^6 PBMC was extrapolated from the number of cells used to isolate DNA for each of the specimens. For low levels of HIV DNA (below 1 copy number per million cells), such as the CD14-negative subsets in Table 3, the values were extrapolated based on the standard curves generated by the iCycler IQ Software Program, version 3.0 (BioRad). Representative graphs

of the β -globin and HIV-1 PCR standard curves and unknown samples are shown in Fig. 3, as described previously [32]. From the β -globin and HIV-1 PCR standard curves, the equivalent cell number and HIV-1 copy number, respectively, are calculated for each unknown specimen. The ratio of HIV DNA copy number per 1×10^6 cells is then acquired. At the lower limits of the standard curves, if limited amount of a specimen is available, the IQ Software Program extrapolated the HIV DNA copy number per 1×10^6 cells, which assumes a linear pattern.

Discussion

The value of proviral DNA levels to assess HIV-1 treatment status has been previously reported, but its utility as a prognostic marker for HAD has not been studied [35,39–42]. While HAART does decrease HIV DNA, the rate of decline is generally lower than its effect on plasma VL [43,44]. HIV DNA plays a role in M/M Φ viral persistence and the levels observed in M/M Φ are generally higher than those found in CD4 lymphocytes [40,45]. In the current study, data are presented that suggest circulating HIV DNA may be an important link in the pathogenesis of HAD. Furthermore, an exploratory evaluation suggests that the greatest burden may be among the CD14/CD16 subsets. It is particularly interesting that the correlation of HAD with circulating HIV DNA remained significant among individuals with undetectable VL, further underscoring that circulating HIV DNA may be involved in pathways leading to HAD.

Neither being treated with HAART, nor achieving undetectable VL, excludes the diagnosis of HAD, as shown by us and others [5,12,24]. While previous studies have shown a variable relationship between HIV DNA level and progression of HIV disease status, we believe the current study is the first to report a link between circulating proviral DNA and HAD [41,46–49]. Our findings are consistent with a model in which M/M Φ harboring HIV DNA are involved in the pathogenesis of HAD. Several mechanisms have been proposed by which M/M Φ might synergistically impact HAD including monocyte trafficking, involvement of perivascular macrophages, and secretion of neurotoxic substances by M/M Φ [2,5,50–56]. This may be partially explained by presence of HIV DNA in M/M Φ . We acknowledge several limitations to the current study, including the inability to confirm that these results are specific to M/M Φ in all individuals with HAD. While an intriguing association was observed suggesting an association between HIV DNA and activated M/M Φ , we were not able to definitively confirm this relationship due to limited specimens. Additionally, the data were obtained by extrapolating and normalizing HIV DNA copy number per 1×10^6 million cells. Therefore, future analyses could benefit by using a more sensitive assay. In the limited subset of patients analyzed, the highest HIV DNA copy number was found within the CD14/CD16 cells and not within the T-cell compartment.

We recognize that while these patients have higher HIV DNA copy numbers in M/M Φ compared to CD4 T cells, the total CD4 counts are also high, therefore the total HIV DNA burden may be significant in these cells. While we have not conclusively shown that the high HIV DNA copy numbers are primarily due to CD14/CD16 monocytes, we feel that our results are highly suggestive and deserve further evaluation. In-depth quantitative studies on suspected target cells would provide insight on the role that proviral DNA plays in the pathogenesis of HAD.

In summary, these data indicate that circulating proviral DNA may be significantly elevated in HAD when compared to HIV-1-seropositive patients with normal cognition, even after controlling for plasma HIV-1 RNA loads. Our future work will determine if proviral DNA represents a sensitive marker to identifying HIV-1-infected individuals at risk for developing HAD or HAD progression; these findings could have therapeutic or prognostic implications.

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Fig. 1. Box plots of HIV DNA

(a) HIV DNA of all individuals ($n = 49$) with NC and HAD. (b) Similar plot of HIV DNA of individuals with undetectable plasma viral levels ($n = 24$). Both plots demonstrate the skewed distribution of HIV DNA while showing the significant difference between the medians when comparing individuals with NC to those with HAD.

Fig. 2. Representative immunofluorescence analysis of CD14/CD16 subset

Dot plot of the purified monocytes (a), which were analyzed prior to CD16 isolation (b). PE, Phycoerythrin; FITC, fluorescein isothiocyanate.

Fig. 3. Representative real-time PCR standard curves with unknown examples

Unknown samples were assayed using β -globin (a) and HIV-1 (b) primers. Samples falling within the standard curve (a) and samples less than the lower limits of the standard curve (b) were analyzed with the iCycler IQ Software Program (BioRad). Samples falling below the lower limits of the standard curve are extrapolated for HIV DNA copy number per million assuming a linear relationship.

Table 1

Demographic data of the study population (n = 49).

Age (years) [mean (SD); range]	45.22 (10.67); 43.07
Years of education	14.35 (2.57); 16.00
Ethnicity (%)	
Caucasian	51
Asian/Pacific Islander	37
Other	12
Sex	
Male	84
Female	16

Table 2

Parameters by diagnostic category.

	Diagnostic category		Odds ratio ^a (P)
	Normal cognition (n = 22)	HAD (n = 27)	
All samples (n = 49)			
HIV DNA (copies/1 × 10 ⁶ PBMC)			
Median (IQR)	0.49 (0.89)	9.11 (37.20)	2.83 (<0.001)
Mean (SD)	2.38 (5.63)	42.9 (118)	
CD4 cell count (× 10 ⁶ /l)			
Median (IQR)	519 (335)	287 (254)	2.76 (<0.01)
Mean (SD)	482 (213)	382 (340)	
Samples with undetectable viral load (n = 24)	(n = 13)	(n = 11)	
HIV DNA (copies/1 × 10 ⁶ PBMC)			
Median (IQR)	0.27 (0.84)	13.20 (48.58)	2.76 (<0.01)
Mean (SD)	1.98 (5.72)	50.4 (181)	
CD4 cell count (× 10 ⁶ /l)			
Median (IQR)	541 (209)	491 (884)	2.76 (<0.01)
Mean (SD)	510 (216)	613 (427)	
Samples with detectable viral load (n = 25)	(n = 9)	(n = 16)	
HIV DNA (copies/1 × 10 ⁶ PBMC)			
Median (IQR)	0.64 (0.86)	7.47 (23.46)	2.76 (<0.01)
Mean (SD)	2.95 (0.86)	17.1 (21.4)	
CD4 cell count (× 10 ⁶ /l)			
Median (IQR)	496 (331)	210 (146)	2.76 (<0.01)
Mean (SD)	442 (215)	224 (116)	

^aOdds ratio in a univariate logistic regression model with HIV-1-associated dementia (HAD) vs. normal cognition as the outcome. The significance remained after controlling for viral load, CD4 cell count, highly active antiretroviral therapy, duration of infection, education, age, and ethnicity. PBMC, Peripheral blood mononuclear cells; IQR, interquartile range.

Table 3

HIV DNA in peripheral blood mononuclear cell (PBMC) subsets.

Patient	Neurocognitive status	White blood cell count ($\times 10^9/l$)	Lymphocytes, monocytes (%)	CD4 cell count ($\times 10^6/l$)	PBMC HIV DNA (copies/ 1×10^6 cells)	HIV DNA of the sorted cells (copies/ 1×10^6 cells)	
						CD14+/CD16+	CD14-
1	HAD	6.6	43, 5	1249	114	110	0.5
2	HAD	6.7	38, 5	1171	618	503	0.01
3	HAD	4.2	37, 6	666	7	5	1
4	Normal	6.3	26, 5	278	2	0.3	1
5	Normal	4.8	30, 6	384	0.3	0.5	0.4

PBMC, Peripheral blood mononuclear cells; HAD, HIV-1-associated dementia.