HIV-associated cognitive disorders: Scientific discoveries through international collaborations in Thailand
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**ABSTRACT**

**Background:** The extent to which highly active antiretroviral therapy (HAART) era cognitive disorders are due to active processes, incomplete clearance of reservoirs, or comorbidities is controversial. This study aimed to determine if immunologic and virologic factors influence cognition after first-time HAART in Thai individuals with HIV-associated dementia (HAD) and Thai individuals without HAD (non-HAD).

**Methods:** Variables were captured longitudinally to determine factors predictive of degree of cognitive recovery after first-time HAART. Neuropsychological data were compared to those of 230 HIV-negative Thai controls.

**Results:** HIV RNA and CD4 lymphocyte counts were not predictive of HAD cross-sectionally or degree of cognitive improvement longitudinally. In contrast, baseline and longitudinal HIV DNA isolated from monocytes correlated to cognitive performance irrespective of plasma HIV RNA and CD4 lymphocyte counts pre-HAART (p<0.001) and at 48 weeks post HAART (p<0.001). Levels exceeding 3.5 log_{10} copies HIV DNA/10^6 monocyte at baseline distinguished all HAD and non-HAD cases (p<0.001). At 48 weeks, monocyte HIV DNA was below the level of detection of our assay (10 copies/10^6 cells) in 15/15 non-HAD compared to only 4/12 HAD cases, despite undetectable plasma HIV RNA in 26/27 cases. Baseline monocyte HIV DNA predicted 48-week cognitive performance on a composite score, independently of concurrent monocyte HIV DNA and CD4 count (p < 0.001).

**Conclusions:** Monocyte HIV DNA level correlates to cognitive performance before highly active antiretroviral therapy (HAART) and 48 weeks after HAART in this cohort and baseline monocyte HIV DNA may predict 48-week cognitive performance. These findings raise the possibility that short-term incomplete cognitive recovery with HAART may represent an active process related to this peripheral reservoir.

**INTRODUCTION**

HIV remains a common cause of dementia internationally while the frequency and severity in countries with broad access to highly active antiretroviral therapy (HAART) has decreased substantially.[1] Early data suggest a failure of HAART to universally eradicate neurocognitive impairment (NCI) in patient populations with access to these treatments.[1-5] Yet controversy exists as to whether continued cognitive impairment following HAART represents an active process, the impact of comorbidities, or pre-HAART brain injury.

In its pure, untreated form, HIV encephalitis is
characterized by CNS immune activation with perivascular macrophage accumulation. It is theorized that CNS injury occurs when monocytes, many of which are infected, transmigrate the blood–brain barrier, resulting in an inflammatory response in the absence of substantial neuronal apoptosis. This pathway has been described in animal models of lentivirus infection. In some animal models, monocyte tissue transmigration appears to trigger gene expression of otherwise quiescent virus, raising the possibility that harboring virus in peripheral monocytes, even when not actively productive, may have clinical consequences upon tissue infiltration.

In this prospective study, we evaluated early immunologic, virologic, and neurocognitive changes with initiation of first-time HAART to clarify the neuropathogenesis of NCI in HAART-treated individuals. We previously reported a correlation between peripheral blood mononuclear cell (PBMC) HIV DNA and NCI in HAART-naïve patients from this cohort. We now identify this relationship specific to circulating monocytes (CD14+ cells) and describe the longitudinal relationship between this marker and cognitive recovery.

METHODS

Patient selection. SEARCH 001 enrolled 15 HIV-infected patients with HAD and 15 without HAD, all confirmed to be infected with HIV-1 circulating recombinant form (CRF) 01_AE. Groups were matched by age, gender, CD4 lymphocyte count, and educational attainment, as previously described. Briefly, individuals were identified from infectious disease clinics, neurology clinics, and HIV testing facilities in Bangkok and were eligible for enrollment if they met Thai national guidelines to initiate HAART and intended to start treatment. All assessments were completed within 1 month before first dose of antiretroviral (ARV) therapy. Participants were hepatitis C antibody negative, denied illicit drug use, lacked factors that would potentially impact cognition (e.g., head injury, learning disability), and had two negative urine toxicology screens on separate days. Normative neuropsychological data were obtained from 230 age- and education-matched HIV-negative Thai controls who denied confounds that could impact cognition (e.g., head injury, medical illness, learning disability, illicit drug use), had a normal screening neurologic examination, were tested to be HIV (EIA) negative, and had a negative urine drug screen.

Cognitive characterization. Dementia was determined by a trained neurologist (P.S.) using standard-of-care assessments in Thailand, which included a comprehensive neurologic examination, simple bedside cognitive testing including the International HIV Dementia Scale (IHDS), patient and proxy reporting of symptoms, and brain MRI. When clinically indicated, lumbar puncture was completed. We employed the neuropsychological testing battery modified from an international HIV battery previously used in Bangkok and designed to minimize cultural influences. Testing was completed by trained nurse-psychometrists with quality assurance review every 6 months.

Cell separation. Peripheral blood mononuclear cells were treated with Accumax (Innovative Cell Technologies, San Diego, CA) to prevent cell clumping. Cells were then washed with RoboSep buffer (Stemcell Technologies, San Diego, CA; PBS, 2% FBS, 1 mM EDTA); counted; centrifuged; and resuspended. Monocyte separation was performed using the monocyte separation kit (Stemcell Technologies). Magnetic bead separation identified monocytes (CD14-positive) and non-monocytes (CD14-negative) fractions, which were then stored at -80°C before HIV DNA analyses.

Proviral HIV DNA assessments. PBMCs were shipped to the University of Hawaii in frozen batches where we isolated DNA from the cells per manufacturer’s instructions (Qiagen, Inc., Valencia, CA) and measured HIV DNA copies. Briefly, we generated standard curves for the real-time PCR assays from dilutions of a plasmid containing one HIV copy (GenBank accession #NC_001802) and one copy of the human housekeeping gene, β-globin (GenBank accession #2253431). Reactions were prepared with 100ng of the sample DNA; HIV gag primers or β-globin primers; 1x iQ supermix (BioRad Laboratories, Hercules, CA); and water to final volume 25μL. Initial denaturation was
performed for 3 minutes followed by 45 cycles of two-step PCR 95°C/10 seconds, 57°C/30 seconds, and a final extension of 72°C/2 minutes. Controls were a negative control (no DNA template) and DNA from three HIV-infected cell lines (8E5, OM10.1, ACH-2, NIH AIDS Research and Reference Reagent Program, Rockville, MD), which were also used for inter-assay calibration. Assays were completed on PBMCs (not monocyte depleted) and monocytes (CD14+ cells) and operators were blinded to HAD status.

**Summary neuropsychological measures.**

Neuropsychological z-scores were calculated by standard methodology compared to appropriate age and educational attainment strata from the normative data. Our initial longitudinal cognitive endpoints were the global deficit score (GDS), a weighted summary of all neuropsychological z-scores, where a higher GDS represents greater impairment and a global non-weighted summary z-score of all tests in our battery (NPZglobal). Preliminary analyses revealed non-significant differences between HAD and non-HAD groups on these measures, despite differences noted in many individuals’ cognitive tests. We therefore formulated a third composite score (NPZcomp) in a post-hoc manner. This was accomplished by contrasting performance characteristics of individual test by HAD/non-HAD group, retaining those at p<0.10, with final selection for those with the largest and most meaningful differences: RAVLT-total of trials 1–5 (learning efficiency), digit symbol modalities task (psychomotor speed), and timed gait (motor speed). A discriminant analysis with cross validation (PROC DISCRIM, SAS) confirmed that the NPZcomp correctly categorized 21/30 (70%) cases as HAD/non-HAD at baseline. We then completed external validation of the NPZcomp in a separate cohort (Hawaii Aging with HIV Cohort, n=286) where the NPZcomp accurately categorized 79% of cases (186/198 non-HAD and 48/88 HAD).

**Informed consent and statistical analyses.** The protocol was approved by the Ethical Review Committees at Phramongkutklao Medical Center, the Walter Reed Army Institute of Research, and the University of Hawaii. All participants signed informed consent. All cases (HAD and non-HAD) were pooled and we employed normal linear regression models to determine the relationship between independent variables and neuropsychological summary scores. All summary scores met model requirements, although the GDS required logarithmic transformation. To take into account within-subject variability, longitudinal mixed normal models were used to assess the effects of baseline levels and subsequent changes in predictive variables (PROC MIXED). In fitting the mixed models, the three cognitive response factors were separately analyzed and correlation of these variables within subjects was assumed to be normally distributed. We employed compound symmetry for our covariance structure, which provided a better fit compared to an unstructured covariance matrix, as assessed by likelihood ratio test. Unless otherwise stated, HIV DNA in the results represents that measured in monocytes.

**RESULTS**

**Baseline immunologic and virologic factors.** SEARCH 001 enrolled 30 individuals and 29 initiated HAART. One case was lost to follow-up and one died before 48 weeks (both in the HAD group). Our analysis included 27 cases with 48-week data available (12 HAD and 15 non-HAD). Factors that distinguished HAD from non-HAD included higher Thai Depression Inventory score (TDI), lower hemoglobin, and higher intracellular PBMC HIV DNA, as previously reported. The relationship between baseline log10 HIV DNA and HAD remained present when analyzed among the CD14-positive subset (monocytes, p<0.001). Plasma HIV RNA level and CD4 lymphocyte count did not correlate to HAD; however, all participants had low CD4 counts (Table 1). The relationship between baseline log10 HIV DNA and HAD remained present when analyzed among the CD14-positive subset (monocytes, p<0.001). Plasma HIV RNA level and CD4 lymphocyte count did not correlate to HAD; however, all participants had low CD4 counts (Table 1). The NPZcomp summary score (p<0.002) and the IHDS (p<0.001) each differed significantly by HAD status. Baseline monocyte HIV DNA correlated to NPZcomp score, but did not meet significance when analyzed for the GDS or NPZglobal measures. The relationship between monocyte HIV DNA and the NPZcomp remained significant after adjustment for plasma HIV RNA and CD4 count ($\beta = -0.253$, SE = 0.059, $p = 0.001$, Figure 1).
48-Week immunologic and virologic response to HAART. One participant’s ARV regimen was not known due to co-enrollment in a second study, which provided HAART in a blinded fashion. All others initiated NNRTI-based ARV with most (21/26) starting nevirapine-based ARV. Among these cases, the NRTI backbone for most (24/26) was stavudine and lamivudine, using GPOvir (fixed-dose combination of lamivudine, stavudine, and nevirapine) manufactured by the Thai Government Pharmaceutical Organization. In general, participants were very adherent with only one having a detectable plasma HIV RNA level at 48 weeks (10,154 copies, non-HAD group). The CD4 lymphocyte counts did not differ between HAD and non-HAD groups at all time points, and at 48 weeks reached a median (interquartile range [IQR]) of 190 (137–234). At 48 weeks, the median log10 monocyte HIV DNA level remained elevated among individuals enrolled into the HAD compared to the non-HAD group, despite suppression of plasma HIV RNA (median [IQR] of 3.09 [0.00–3.94] for HAD and 0 [0–0] for non-HAD, p < 0.001, Figure 2). The rate of decline in log10 monocyte HIV DNA was similar in both groups during the first 24 weeks (p=0.498), but greater in the HAD group (β =0.157, SE = 0.05) compared to the non-HAD group (β =0.017, SE=0.026, p=0.035) between weeks 24 and 48, reflecting a floor effect in the non-HAD group. Only 4/12 individuals enrolled into the HAD group compared to 15/15 individuals in the non-HAD group were able to lower their monocyte HIV DNA level to below the limit of detection of our assay (10 copies/106 cells) at 48 weeks.

48-Week cognitive response to HAART. Both the HAD and non-HAD groups exhibited a robust cognitive response to HAART (Figure 3). Combining groups, the 48-week change in all composite measures was significant (NPZglobal: from -0.430 to +0.063, p<0.001; NPZcomp: from -0.619 to +0.299, p=0.001; log10 GDS: from 0.573 to 0.228, p <0.001). However, at 48 weeks, the
HAD group continued to have poorer performance on the NPZcomp (p<0.001) but not on the NPZglobal (p=0.205) or log\textsubscript{10} GDS (p=0.132). We did not identify an interaction effect by group on change in these measures (NPZcomp, p=0.721; NPZglobal, p=0.748). The performance approached that of controls in the HAD group and appeared to exceed that of controls in the non-HAD group; however, learning effects were not factored in as only cross-sectional normative neuropsychological data were available.

Most individual cases also exhibited a beneficial cognitive response to HAART, with one notable exception. This HAD individual underwent additional evaluations finding no opportunistic disease and both plasma and CSF HIV RNA levels that were undetectable (<50 copies/mL). This participant’s PBMC HIV DNA remained relatively high at 48 weeks (baseline: 3.39 log\textsubscript{10} HIV DNA/106 PBMC; week 48: 2.64 log\textsubscript{10} HIV DNA/106 PBMC); although monocyte HIV DNA was below the level of detection of our assay at 48 weeks. At subsequent follow-up (6 months), both monocyte (CD14+) and PBMC HIV DNA were detectable in this case.

Predictors of cognitive response to HAART. Baseline monocyte HIV DNA was predictive of 48-week NPZcomp score (p<0.001, Table 2). The relationship between monocyte HIV DNA and the NPZcomp score remained present in a multivariate model that included age, education, TDI, and baseline CD4 count (β=-0.238, SE=0.073, and p<0.004). In mixed models, the change in log\textsubscript{10} monocyte HIV DNA correlated with change in NPZcomp in a model that include baseline log\textsubscript{10} monocyte HIV DNA and baseline NPZcomp (β=-0.171, SE=0.041, and p<0.001). A decrease of one log\textsubscript{10} HIV DNA was associated with a 0.171 increase in NPZcomp. Baseline log\textsubscript{10} monocyte HIV DNA level was also independently predictive of 48-week performance on the NPZcomp (β=-0.257, SE=0.047, and p<0.001). Change in log\textsubscript{10} monocyte HIV DNA level did not correlate with change in our other neuropsychological summary scores (p=0.272 for NPZglobal and p=0.243 for log\textsubscript{10} GDS).

DISCUSSION

This report confirms cognitive improvement with HAART among individuals from Southeast Asia and, specifically, among individuals known to be infected with HIV-1 CRF 01_AE. This finding complements our understanding of cognitive improvement in subtype B infected individuals\textsuperscript{20} and more recent reports among patients presumed to be infected with subtypes A and D in Africa.\textsuperscript{21} We also report that HIV DNA isolated specifically from circulating monocytes correlates to HAD in
HAART-naïve individuals. In this cohort, having a monocyte HIV DNA level exceeding 3.5 log_{10} copies/10^6 cells identified all HAD and excluded all non-HAD cases, indicating 100% sensitivity and specificity for HAD in this small cohort. Monocyte HIV DNA also relates to cognition 48 weeks post HAART, and, while less clear, baseline monocyte HIV DNA may predict short-term (48-week) cognitive response to HAART. This finding is more notable in light of our previous work with this cohort, which failed to identify the cell surface activation markers CD14+/16+ as predictive of HAD.

The lack of correlation between our a priori cognitive measures (GDS and NPZglobal scores) and HAD at baseline was unexpected and is likely due to the small sample size and diminished sensitivity inherent in pooling measures. We cannot rule out the potential influence of selection bias associated with the tools used to diagnose HAD. Cultural influences could be a factor despite choosing a battery designed to minimize such bias. Consequently, our certainty regarding the impact of HIV DNA on cognitive improvement is diminished since we required development of the NPZcomp measure in a post hoc manner. Nevertheless, the NPZcomp was designed using robust statistical models to capture HAD status at baseline in this cohort, was validated externally, and represents cognitive tests with validity in HIV.

Our findings extend our knowledge of HAD in HAART-treated individuals by noting an incomplete cognitive recovery in some individuals at 48 weeks and suggesting that this incomplete recovery may in part be an active process. While HAART suppresses plasma HIV RNA in most individuals and induces immunologic recovery, treatment does not eradicate virus within reservoirs, such as monocytes. In our cohort, only four HAD cases but all non-HAD cases decreased monocyte HIV DNA levels to below that detectable by our assay (10 copies/10^6 cells) at 48 weeks. The extent to which longer duration of treatment with HAART will further diminish this reservoir is not yet known, but is expected based on published work. Full clearance may not occur in all individuals with typical HAART based on our previous cross-sectional work where patients with HAD, many of whom had been on stable HAART for years and with undetectable plasma HIV RNA, were more likely to have elevated PBMC HIV DNA.

The accumulated evidence suggests that monocyte HIV DNA plays a role in HIV neuropathogenesis and raises the possibility that, in some patients, incomplete cognitive recovery with HAART may
be an active process associated with this peripheral reservoir. Previously described fluctuation in cognition also supports an active process, although comorbidity may account for some of this fluctuation. Comorbidity would less likely explain variability in our cohort as individuals were meticulously screened for confounding factors and highly adherent to ARV. Most were young and none had brain opportunistic infection or hepatitis C. All denied illicit drug use and were tested negative twice by urine toxicology screen. In our cohort, the mean TDI scores were higher in HAD cases; however, all cases of major depression were excluded. Nevertheless, depressive symptoms must be considered to potentially impact our assessments. The overlap between the symptoms of HAD (slowed responses, flat affect, and apathy) and depressive symptoms is well described.

Our findings are consistent with existing models of monocyte transmigration resulting in CNS infection and inflammation. They are also congruent with reports that HIV DNA influences HIV disease progression. For example, elevated HIV DNA was reportedly predictive of poor response to antiretroviral therapy in early studies and of patients who experienced virologic failure in another study. HIV DNA predicts progression to AIDS in the SEROCO cohort study in Europe, independently of plasma HIV RNA and CD4 counts, and in the PRIMO Cohort, where patients are enrolled at the time of primary HIV infection. In our work, it is not clear if HIV DNA in circulating monocytes exhibits its effects through elevation of CSF HIV RNA; however, one patient with elevated 48-week PBMC HIV DNA and cognitive impairment had undetectable CSF HIV RNA (limit of detection, 50 copies/mL). We predict that this marker’s impact is independent of CSF HIV RNA.

REFERENCES


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Dr. Valcour is a consultant for GlaxoSmithKline.
The opinions expressed herein are those of the authors and do not represent the views of the Department of the Army or the Department of Defense.

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