Pleocytosis is associated with disruption of HIV compartmentalization between blood and cerebral spinal fluid viral populations

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A B S T R A C T

Introduction: We hypothesized that pleocytosis, which is a marker of central nervous system (CNS) inflammation, would result in viral genetic equilibration or de-compartmentalization between HIV populations in the blood and cerebrospinal fluid (CSF), suggesting viral trafficking.

Methods: Study subjects, who started or interrupted their antiretroviral treatment, had viral loads measured and clonal viral env sequences generated from HIV RNA extracted from paired blood and CSF samples. White blood counts in CSF were also measured at each timepoint. Degree of inter-compartment segregation was calculated by posterior probability using linear discriminant analysis and multidimensional scaling. Co-receptor usage was determined using a trained support vector machine.

Results: Pleocytosis was strongly associated with disruption of viral compartmentalization.

Conclusions: Inflammation in the CNS, marked by pleocytosis, allows HIV populations to mix between blood and CSF, which may increase the overall viral genetic diversity within the CSF.

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Introduction

HIV-1 invades the central nervous system (CNS) early during primary infection, and can cause neurological disease ranging from minor cognitive and motor disorders to severe dementia. Neurocognitive manifestations of HIV remain a persistent therapeutic problem in the current era of potent antiretroviral drugs, as 50% of persons with HIV and CD4 counts <500/ml have at least mild cognitive impairment and nearly 7% of patients with AIDS develop HIV associated dementia.

The CNS differs from other tissues in immunological surveillance, cytokine milieu, target cell characteristics, and antiretroviral penetration. Such an environment provides a unique reservoir for HIV-1 replication. Viral replication and evolution within the CNS most likely can lead to mutational adaptation to neuronal tissues, and phylogenetic analyses have documented distinct subpopulations in the blood and CNS compartments. Genetic variants adapted to the CNS may be the basis of at least some of the HIV associated neurocognitive dysfunction (HAND). The causes of HAND are most likely multifactorial, and may include direct toxic effects of HIV proteins, cytokine mediated damage, host genetic susceptibility, added inflammatory effects from other CNS tropic infections, like syphilis, Cryptococcus or herpes viruses.

To determine how CNS inflammation can change the viral dynamics in the CNS, we investigated how viral genetic compartmentalization between blood and CSF changed in the setting of pleocytosis. Pleocytosis is the appearance of leukocytes in the CSF, which is used clinically as a marker of meningeal inflammation and is defined as >5 cells/mm³. Pleocytosis can occur intermittently during HIV-1 infection and most likely reflects an interruption of the blood–brain barrier and trafficking of white blood cells from the blood into the CSF. We investigated if such a barrier disruption may provide an opportunity for infected lymphocytes to migrate into the CSF and thus introduce blood-derived viruses into the CNS. As a result, we would expect to identify a disruption of viral compartmentalization between the CNS and blood during periods of pleocytosis. Using clonal sequence analysis of HIV RNA viral populations on longitudinally collected paired blood and CSF samples, we studied the relationships between pleocytosis and degree of viral compartmentalization and inter-compartment correlation between blood and CSF.

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Results

Study population

All study participants lived in San Diego and reported sex with men as their only HIV risk factor. The average age of the participants at enrollment was 47 years (range 34–58 years), the average CD4 count at study enrollment was 306 cells/ml (range 200 to 461 cells/ml), and none had evidence of active systemic or CNS opportunistic infections or malignancies during the study. Participants were sampled either before or after interrupting antiretroviral therapy (n = 3, subjects 839, 409, and 487) or before and after starting therapy (n = 1, subject 686) (Fig. 1). Regimens of antiretroviral therapy for each participant included at least three antiretrovirals from two different classes. Although the number of participants was small in each group, there were no statistical differences in baseline CD4 or CSF or blood viral loads between participants who demonstrated minimal (subjects 686) or persistent (subject 487) pleocytosis or those participants who developed pleocytosis (subjects 839 and 409) (data not shown).

Association between viral compartmentalization and pleocytosis

Viral compartmentalization was determined using two approaches: Slatkin–Maddison test, which has been shown to be robust in detecting within patient compartmentalization of HIV populations and linear discriminant analysis. In one participant (686) minimal pleocytosis was demonstrated, viral compartmentalization between the blood and CSF derived viral populations persisted throughout the study period (Table 1); viral sequences clustered strictly by anatomic compartment and not by time of sampling by both phylogenetic analysis (Fig. 2) and multidimensional scaling (Table 1) of clonal env sequences at each timepoint. Two other participants (subjects 839 and 409) had no pleocytosis at the beginning of the study period and compartmentalization persisted in both the blood and CSF. When pleocytosis developed, however, the observed compartmentalization was disrupted and was negatively correlated with the level of leucocytes in the CSF for all sampled timepoints for subject 839 and for the first and second timepoint samples for subject 409. This is exemplified for subject 839 in Figs. 3A–C. Subject 409 was unique among the study group; he demonstrated significant viral compartmentalization (Slatkin–Maddison test: p < 0.001) during a high level of pleocytosis (WBC 101) at the third time point sampling. This sample was also unique among all patient samples for CSF viral loads being higher than blood viral loads at the time of sampling. In one participant (subject 487), leucocytes were persistently elevated in the CSF after stopping antiretroviral therapy and during sampling (Table 1). Viral compartmentalization between blood- and CSF-derived viral isolates was not observed in these samples (Table 1). Overall, viral compartmentalization was significantly associated with the level of white blood cells measured in the CSF (p = 0.03).
across participants. Although previously described in larger cohorts, no amino acid residues were found to vary significantly across all participants between blood and CSF whether or not anatomic compartmentalization of viral populations was identified.

**Genetic diversity**

Viral genetic diversity did not correlate with the degree of pleocytosis in either the CSF or the blood across patients \( (p > 0.1) \). Within subject viral diversity also did not change appreciably in the compartment in relation to CSF WBC for all participants; however, viral genetic diversity did increase in the CSF in subjects 839 and 409 when pleocytosis developed \( (p < 0.05) \). Viral genetic diversity did not change in CSF in relation to the level of CSF WBC for subject 487, who had relatively high levels of pleocytosis throughout the sampled timepoints. Subject 686 had a trend in decreasing diversity during a slight increase in pleocytosis \( (p = 0.07, 1 \text{ to } 7 \text{ cells/ml}) \) (Table 1).

**Co-receptor usage**

Based on genotypic analysis, all virus isolates from all subjects, except subject 686, exclusively used the CCR5 co-receptor, regardless of whether they were derived from the blood or CSF. For subject 686, viral isolates that used CXCR4 were exclusively found in the third population in the blood and the small proportion of blood sampled for the CNS, which may mediate direct or indirect neurotoxic effects without genetic changes in the local virus. On the other hand, CSF pleocytosis may influence neuroadaptation and associated neurovirulence by trafficking HIV-infected lymphocytes into the CNS, potentially increasing CNS viral genetic diversity. Alternatively, increased viral trafficking during pleocytosis would increase the concentration of viral proteins in the CNS, which may mediate direct or indirect neurotoxic effects without genetic changes in the local virus. On the other hand, CSF pleocytosis and associated disruption of viral compartmentalization may not have a direct effect on HAND at all, but may only serve as a marker of CNS inflammation, and the chronic nature of HIV infection seems to exert insidious and persistent effects on the CNS often culminating in symptomatic HAND in the later stages. Possible mechanisms for this include neuroadaptation and associated neuropathogenicity, and neuroinflammation; however, these processes are not necessarily exclusive from each other. Pleocytosis may influence viral neuroadaptation and associated neuropathogenicity by trafficking HIV-infected lymphocytes into the CNS, potentially increasing CNS viral genetic diversity. Alternatively, increased viral trafficking during pleocytosis would increase the concentration of viral proteins in the CNS, which may mediate direct or indirect neurotoxic effects without genetic changes in the local virus. On the other hand, CSF pleocytosis and associated disruption of viral compartmentalization may not have a direct effect on HAND at all, but may only serve as a marker of CNS inflammation, and inflammation itself may be the destructive force that results in HAND. Because of the small number of participants in this study, HAND was not assessed in relation to pleocytosis or viral compartmentalization; therefore, it remains unclear to what degree HAND is associated with persistent or intermittent inflammation and to what extent CNS inflammation is the mechanism underlying HAND. Also, given the small nature of our study, we were unable to assess other important processes, like stochastic and founder effects and host immune responses that are likely to influence the entire gamut of the inter-related factors of pleocytosis, compartmentalization, intra-host evolution, neuroadaptation, and neurovirulence; therefore, future studies such as these are needed to further delineate the mechanisms of HIV-mediated neuropathogenesis.

**Methods**

**Study participants, specimen collection and clinical data**

Paired blood and CSF samples were collected from four male participants, who were enrolled in longitudinal clinical studies at the University California San Diego HIV Neurobehavioral Research Center (HNRC) between years 1997 and 2001. All participants were selected based on measurements of CSF pleocytosis before or after starting or
interrupting antiretroviral therapy (Fig. 2). One participant (subject 686) was selected because he had minimal evidence of pleocytosis (peak <8 cells/ml of CSF) at all timepoint samples. Two other participants (subjects 839 and 409) were selected because they developed pleocytosis at some timepoints (peak 17 and 185 cells/ml of CSF respectively). One participant (subject 487) was chosen because he demonstrated pleocytosis at all timepoint samples (range 16–185 cells/ml of CSF). None of the patients had evidence of systemic or CNS opportunistic infections or malignancy based on clinical, laboratory and neuro-imaging studies. Data were available on past and present medical therapy, current viral load and CD4 counts, nadir CD4 counts and CSF cell counts. All studies were conducted in compliance with local IRB guidelines and with written informed consents. Paired blood from peripheral venipuncture and CSF from lumbar punctures were collected and processed within 2 h. Blood plasma and cell free CSF were aliquoted and frozen and stored at −80 °C until processing.

HIV RNA extraction and quantitation

HIV RNA was extracted and quantified from 500 μl of blood plasma per manufacturer’s protocol (50 copies/ml minimal level of detection; Amplicor HIV-1 Monitor™ Test, Roche Molecular Diagnostics, Pleasanton, California, USA). HIV RNA was extracted from 1 ml of CSF and then quantified (50 copies/ml minimal level of detection; Amplicor HIV-1 Monitor™ Test, Roche Molecular Diagnostics, Pleasanton, California, USA), as previously described.

ev sequencings

Reverse transcription and PCR amplification of C2-V3 env for HIV RNA from each sample was performed in triplicate using the Finnzyme one step RT-PCR kit (MJ Research), and primers V3Fout and V3Bout as previously described in a 50 μl reaction volume. 5 μl of first step RT-PCR product was used in the second, nested PCR reaction with primers V3Fin and V3Bin, Tris pH 8.0, 30 pmol each primer, 2.5 mM MgCl, 200 μM dNTP, BSA 10 mg/ml in a total volume of 50 μl. Cycling parameters were: 94 °C×2 min; 94 °C×30 s, 54 °C×30 s, 72 °C×1 min for 35 cycles; 72 °C×10 min. All assays were conducted in conditions to minimize PCR contamination utilizing aerosol resistant pipet tips, dedicated PCR reagents and laminar flow hoods. All assays included negative controls. Blood plasma and CSF samples were processed separately on different days to minimize within-patient cross contamination of samples. Nested PCR products were visualized by agarose gel electrophoresis and ethidium staining. Replicate PCR products were proportionately pooled and cloned using the TOPO-TA™ cloning system (Invitrogen, Carlsbad, CA). Twenty clones were selected by blue white screening and expanded in 1.5 ml broth cultures. Plasmid clones were purified using Qiagen Mini-prep kits (Qiagen, Chatsworth, CA). Purified plasmids were sequenced bidirectionally with –20M13 primer (5′-sequence-3′) or TOPO Forward primer (5′-sequence-3′) using Prism Dye terminator kits (ABI, Foster City, CA) on an ABI 3100 Genetic Analyzer. Sequences were initially compiled, aligned, and manually edited using Sequencher™ 4.0 (GeneCodes, Ann Arbor, MI). Preliminary alignments were then performed using the MUSCLE package. Generated alignments were then inspected and further aligned manually using Bioedit. Sequences are publicly available at Genbank (accession numbers FJ159856–FJ160261).

Fig. 3. (A) Phylogenetic Analysis of viral sequences derived from blood and CSF from subject 839 who demonstrated no pleocytosis (3 WBC/ml) at timepoint 1. Legend: C2-V3 env sequences derived from blood (closed circles) and CSF (open circles) of subject 839 were phylogenetically distinct at the first timepoint, p<0.01 per Slatkin Maddison test. (B) Phylogenetic Analysis of viral sequences derived from blood and CSF from subject 839 who had developed pleocytosis from timepoint 1 (1 WBC/ml) to timepoint 2 (15 WBC/ml). Legend: C2-V3 env sequences derived from blood (closed triangles) and CSF (open triangles) of subject 839 were not phylogenetically distinct at the second timepoint, p>0.5 per Slatkin Maddison test. (C) Phylogenetic analysis of viral sequences derived from blood and CSF from subject 839 where pleocytosis persisted from timepoint 2 (15 WBC/ml) to timepoint 3 (17 WBC/ml). Legend: C2-V3 env sequences derived from blood (closed squares) and CSF (open squares) of subject 839 were not phylogenetically distinct at the third timepoint, p>0.05 per Slatkin–Maddison test.
Phylogenetic and genetic distance and diversity analysis was performed using HyPhy (T/T ratio of 2.0, gamma distributed substitution rate) on clonal sequences for individual subjects and the timepoint and compartment sampled, and DNADIST and NEIGHBOR methods were used to reconstruct the phylogeny of the entire dataset. Trees were displayed using Treeview. Assessment of degree of intercompartment segregation was performed by testing for Panmixis using gene phylogenies as implemented in MacClade. In brief, from the maximum likelihood trees for each individual patient’s 2C3 sequences and their characterization according to compartment of origin, the minimum number of intercompartment migration events allowed by the tree was tallied. This result was compared to the distribution of migration events for 1000 randomly generated trees. Evidence of restricted gene flow (compartamentalization) was documented when <1% (or between 1% and 5%) of the random trees required the same or a fewer number of migration events as for the sample data. Genetic diversity analyses were also performed in relation to the degree of pleocytosis across and within subjects, and across and within anatomic compartments.

Analysis of sequence differences between CSF and blood virus

Prediction of co-receptor usage was performed using the Wetcat SVM (support vector machine), available at: http://spots.ucsd.edu:8080/wetcat. Residues at which amino acid frequencies varied significantly between compartments were identified by Monte Carlo approximation of the Mantel-Haenszel and likelihood ratio tests, based on identity of individual amino acid or on chemical class of the amino acid residue (positive, negative, neutral or hydrophobic) with p < 0.05 considered to be significant. Residue positions demonstrating intercompartmental variation by both of these univariate measures were included in a stepwise logistic regression (with entry cutoff p < 0.10 and retention cutoff p < 0.05). Forward logistic regression was performed with the entry criterion p < 0.05. Analyses were performed on the entire dataset as well as on “pruned” data including only those patients exhibiting phylogenetic segregation of CSF-and blood-derived sequences verified to be significant by the Slatkin–Maddison test (p < 0.01) and in which CSF-derived sequences were not intermingled with significant numbers of blood-derived sequences, i.e. viral compartmentalization was observed. Residue specific entropy was computed from the frequency f (A)_i of amino acid A at position i according to \(-\sum f(A) \ln[f(A)]\).

Multidimensional scaling, linear discriminant and statistical analysis

For each patient and timepoint a euclidean distance matrix was built using intra-patient sequence data, with the aid of the software package ape. These distance measures were represented in two dimensions through multidimensional scaling using the software package MASS. Linear discriminant analyses were further carried out to classify the viruses as belonging to the blood or the CSF compartment, and posterior probabilities were calculated for leave-one-out cross-validation, using the package MASS. Subsequent analyses were performed using the analytical package R. For parameters such as CD4 count, CSF pleocytosis and viral load, both parametric (ANOVA and F test) and non-parametric (Kruskal–Wallis and Wilcoxon Rank) tests were applied after log transformation of viral load data and square root transformation of CD4 count data. Significance was defined at the p < 0.05 level. Statistical analyses were performed using SAS V8 and JMP IN (SAS Institute, Cary NC).

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