Dynamics of Simian Immunodeficiency Virus Populations in Blood and Cerebrospinal Fluid over the Full Course of Infection

Patrick R. Harrington,1,* Mary J. Connell,4 Rick B. Meeker,3 Philip R. Johnson,4 and Ronald Swanstrom1,2

1Lineberger Comprehensive Cancer Center, 2Center for AIDS Research, Department of Biochemistry and Biophysics, and 3Department of Neurology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill; 4The Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania

Background. Human immunodeficiency virus (HIV) replication and compartmentalization in the central nervous system, including in cerebrospinal fluid (CSF), are associated with severe neurological disease and may contribute to viral persistence during antiretroviral therapy. To understand the relationships between viral populations in multiple compartments, we performed a systematic longitudinal characterization of viral populations in blood plasma and CSF obtained at short time intervals over the full course of infection in 3 macaques infected with simian immunodeficiency virus (SIVsm strain E660).

Methods. Complex viral genetic populations in blood plasma and CSF were characterized using a heteroduplex tracking assay targeted to the V1/V2 hypervariable region of env. To identify signs of neurological disease, monocyte chemoattractant protein (MCP)–1 levels in CSF and CD68+ monocyte/macrophage infiltration in brain tissues were quantified.

Results. Two patterns of blood/CSF viral dynamics were apparent as infection progressed: concordant blood/CSF viral evolution and discordant blood/CSF viral evolution. Perivascular CD68+ cells in autopsy brain tissue and elevated CSF MCP-1 levels accompanied blood/CSF viral population discordance but not concordance.

Conclusions. Two distinct patterns of blood/CSF viral population dynamics can be observed in SIV-infected macaques, and the patterns may be associated with different neurological disease outcomes.
Studies of neuropathogenesis often focus on characterizations of HIV-1 populations and host factors in CSF, which can be sampled repeatedly with minimal risk. Virus persists in the CSF compartment throughout the course of infection in the absence of antiretroviral therapy [7–12]. The precise source of CSF HIV-1 is unclear but may include infected lymphocytes or monocytes trafficking from the periphery into the CNS, cell-free virus trafficking from the periphery into the CNS, and/or infected cells residing in the CNS [13–18]. Numerous cross-sectional genetic comparisons of HIV-1 in blood and CSF have revealed that these viral populations are often different [18–21], or compartmentalized, suggesting that at least a portion of the CSF HIV-1 population may be genetically adapted to and produced locally within the CNS. However, the kinetics and dynamics of viral compartmentalization in CSF throughout infection are unclear, because no study has monitored evolving blood/CSF viral populations longitudinally at short intervals for an extended period.

The SIVsm strain E660 recapitulates many aspects of the typical disease course in HIV-infected humans, including the viral population dynamics in blood and the incidence and severity of CNS disease. Like HIV-1, SIVsm E660 uses the CCR5 coreceptor to infect both macrophages and lymphocytes, invades the CSF compartment during primary infection, establishes an asymptomatic phase of infection that can last for months to years before immune deficiency and end-stage disease occur, and ultimately causes a dementia-like illness in approximately one-third of infected macaques [22, 23]. In the present study, we used the heteroduplex tracking assay (HTA) to characterize blood and CSF viral genetic populations over the full course of disease in macaques infected with SIVsm E660 and demonstrated that SIVsm E660 invades the CSF compartment early during infection, with little evidence of a selective viral genetic barrier between blood and CSF. We observed 2 distinct patterns of viral population dynamics in blood and CSF as infection progressed—one in which the blood and CSF viral populations were almost completely concordant throughout infection, and another in which the viral populations were discordant. Finally, we provide preliminary evidence suggesting that the distinct blood and CSF viral population patterns may be associated with different neurological outcomes.

MATERIALS AND METHODS

Virus, animals, and specimen collection. The SIVsm E660 challenge inoculum was previously isolated from an infected rhesus macaque [24]. Four macaques were challenged intravenously (iv) via the sapheneous vein with ∼50 ID₅₀ U of SIVsm E660 (i.e., 50 times the 50% macaque infectious dose). Blood plasma and CSF specimens were collected (on the same day) before challenge, every 2 weeks for approximately the first 3 months of infection, and every 4 weeks thereafter. No CSF specimens with evidence of blood contamination were used. Infected macaques were euthanized at end-stage disease, as determined by severe weight loss, diarrhea, and/or wasting. One macaque (C123) that apparently remained uninfected was killed ∼8 months into the study and included as a negative control animal. Blood, CSF, and brain tissues were harvested at autopsy and immediately fixed or frozen. All animal care was performed at the Columbus, Ohio, Children's Research Institute in accordance with institutional guidelines.

RNA isolation, reverse-transcription polymerase chain reaction (RT-PCR), and HTA. Isolation of viral RNA from blood plasma and CSF, RT-PCR targeting the V1/V2 region of env, and V1/V2 env HTA were performed as described elsewhere [25]. Briefly, viral RNA was extracted using the QIAamp viral RNA extraction kit according to the manufacturer’s instructions (Qiagen). To increase the number of viral RNA templates from CSF for RT-PCR, 0.5–1 mL of CSF specimens was first centrifuged at low speed to remove any cells; virus was then pelleted in the cell-free CSF by centrifugation at 25,000 g and the entire virus pellet was subjected to RNA extraction. Approximately one-fifth of the RNA extractions were subjected to RT-PCR targeting the V1/V2 region by use of a 1-step RT-PCR kit (Qiagen), and the RT-PCR products were then analyzed by HTA using a single-stranded, ³⁵S-labeled V1/V2 DNA probe based on the SIVsm H4 molecular clone [25, 26]. Two independent RT-PCR amplifications and HTA analyses were performed for each blood plasma and CSF specimen, to validate the sampling quality of the complex viral genetic populations. All data reported were obtained only from specimens that yielded reproducible HTA band patterns, which is indicative of efficient sampling of complex viral genetic populations independent of viral RNA load (VL).

HTA data analyses. Dried HTA gels were exposed to phosphorimaging screens, and the relative migration and abundance of HTA bands were measured using a phosphorimager and ImageQuant TL software (GE Healthcare). Two different analyses of the V1/V2 HTA results were performed: (1) the percentage of divergence from the initial population and (2) the percentage of plasma/CSF discordance [20, 25, 27, 28]. The percentage of divergence measurements were used to quantify the amount of viral population change over time relative to the viral population that initiated infection, on the basis of baseline sampling done either 14 or 15 days after challenge. The percentage of divergence from the initial population for
each sampling time point after day 14 or 15 was calculated using the following formula:

\[
\% \text{ of divergence} = 100 \times \frac{1}{n} \sum_{i=1}^{n} |B_i - S_i|
\]

where \( B_i \) is the relative abundance of a baseline band that migrates to a particular point on the gel, \( i \), for all bands, \( n \), and \( S_i \) is the relative abundance of a band migrating to the same position for a subsequent time point. For bands that are detected at one time point and not the other, the undetectable band is assigned a relative abundance of 0. The sum of the absolute difference for all bands is divided by \( n \) because an increase in abundance of one band reflects a concomitant decrease in the abundance of another and is multiplied by 100 to obtain a percentage value. A percentage of divergence background level was determined by calculating the percentage of divergence between individual HTA replicates of baseline blood plasma viral populations. Percentage of blood/CSF discordance was similarly calculated by measuring differences in the relative abundance of distinct band migrations in blood and CSF HTA lanes rather than the baseline ground level was determined by calculating the percentage of divergence back.

\[
p \% \text{ of divergence} = 100 \times \frac{1}{n} \sum_{i=1}^{n} |B_i - S_i|
\]

\( p \) is the relative abundance of a band migrating to the same position of baseline (B) and subsequent (S) HTA lanes shown in the equation above.

**CNS disease markers.** At necropsy, the brain was split sagitally along the midline; half of the brain was frozen, and the other half was placed in buffered formaldehyde. The caudate nucleus adjacent to the lateral ventricle and the overlying cortex were dissected from the brain and embedded in paraffin. Coronal paraffin sections were cut from these blocks and included most of the basal ganglia and frontal cortex. Sections extended laterally from the wall of the lateral ventricle and included the caudate nucleus and portions of the putamen. Sections of the adjacent frontal cortex and underlying white matter were also cut and processed in parallel. The sections were deparaffinized 3 times in xylene for 10 min, followed by rehydration through a descending series of alcohols and water. Macrophages were stained using the ABC system (Vector Laboratories) according to the manufacturer’s guidelines. Sections were blocked with horse serum in 0.01 mol/L phosphate buffer in saline (0.9% NaCl). Monocytes and macrophages were identified by overnight staining at 4°C with a mouse monoclonal anti–human CD68 antibody (1:100 in PBS) (Dako). The primary antibody was detected using an avidin-conjugated universal anti-mouse secondary antibody (1:250 in PBS). Sections were then washed in PBS, incubated in Elite ABC reagent (Vector Laboratories) for 60 min, washed in PBS, and developed with enhanced diaminobenzidine reagent (Pierce). The sections were rinsed in dH2O, counterstained with Giemsa, dehydrated, and mounted on coverslips by use of Permount (Fisher Scientific). Stained sections were examined and images were captured with a digital camera and Metamorph imaging software (version 5.0r6; Universal Imaging).

Monocyte chemoattractant protein (MCP)–1 levels in CSF were quantified using a sandwich ELISA kit for detecting human MCP-1 (R&D Systems).

**RESULTS**

**Characterization of systemic infection in macaques challenged with SIVsm E660.** Four macaques were iv exposed to 50 ID50 doses of SIVsm E660. All 4 macaques underwent an intense schedule of blood and CSF collection to characterize and compare viral populations in these compartments. Specimens were collected every 2 weeks for approximately the first 3 months and then approximately every 4 weeks until end-stage disease and euthanasia.

Viral infection was monitored over time on the basis of blood plasma VL (figure 1). Three of the macaques became infected, with blood plasma VL peaks of \( \sim 1 \times 10^3 \) copies/mL and with VL set points of \( \sim 1 \times 10^2 \)–\( 1 \times 10^3 \) copies/mL. One challenged macaque, C123, was apparently not infected; this animal was included in subsequent analyses as a negative control. All 3 infected macaques progressed to end-stage disease and were euthanized 170–300 days after challenge. The CSF VL was also determined for a few sampling time points, although the limited volume of CSF precluded a detailed analysis of VL in this compartment, because it was necessary to preserve sufficient material for characterization of viral genetic populations. Nevertheless, the limited VL data and observations of PCR robustness suggested that the CSF VL was generally lower than the blood plasma VL in all macaques (data not shown).

**Viral population dynamics in blood.** We used HTA tar-
Figure 2. Viral population dynamics in blood plasma. In panel A, viral genetic populations in blood plasma were resolved by V1/V2 env heteroduplex tracking assay (HTA). The top band present in all lanes represents single-stranded, 35S-labeled probe, and the bottom band represents probe annealed to its unlabeled, 100% complementary strand. All other bands represent heteroduplex populations. An asterisk indicates a necropsy specimen. The efficiency of viral RNA template sampling was validated for all lanes shown by confirming the reproducibility of band patterns in independent reverse-transcription polymerase chain reaction amplifications and HTA analyses. In panel B, lanes were scanned with a phosphorimager to measure the migration and relative abundance of individual heteroduplex bands, which were then used to quantify viral population genetic divergence (percentage of divergence) from the baseline populations (day 14 or 15). The percentage of divergence of 2 individual HTA replicates for the day 14 or 15 time points was calculated to illustrate HTA reproducibility and to provide a percentage of divergence background level. Values for all other time points reflect the average percentage of divergence from baseline for 2 HTA replicates, with error bars indicating SDs.

We first characterized the dynamics of viral RNA populations in the peripheral blood plasma of the 3 infected macaques by V1/V2 env HTA (figure 2). The HTA data were analyzed using a phosphorimager and ImageQuant software to measure the migration and to quantify the abundance of distinct viral subpopulations. These analyses were then used to quantify viral population divergence from the initiating viral population at day 14 or 15 after challenge (percentage of divergence), as described elsewhere [25].

Analysis of the SIVsm E660 inoculum revealed a swarm of at least 5 detectable V1/V2 genetic variants, with multiple variants transmitted to each macaque (figure 2A). In all 3 infected macaques, the HTA patterns remained relatively stable for the first 1–2 months after challenge, although changes in the relative abundance of subpopulations were apparent early during infection. Dramatic changes in the HTA patterns occurred after this initial period of relative stability in macaques D110 and C002. In these animals, the percentage of divergence reached 100% by end-stage disease, reflecting the replacement of the entire initiating population by newly emerged genetic variants (figure 2B). Changes in the V1/V2 population in macaque D081 were also observed, including shifts in the relative abundance of persisting variants as well as the eventual disappearance of the variant that was most abundant at day 14. However, these changes were less striking overall relative to those in macaques D110 and C002.

We also characterized viral population complexity (Shannon entropy) and neutralizing antibody titers over time against a neutralization-sensitive SIVsm variant, SIVsm H4. Consistent with previous observations from SIV-infected macaques and HIV-infected humans [25, 33–35], neutralizing antibody titers in the macaques increased concurrently with the divergence and expansion of V1/V2 genetic populations (data not shown). Taken together, the course of systemic infection in all 3 macaques progressed as typically observed in macaques challenged with SIVsm E660 [22, 23, 25].
Figure 3. Viral population discordance in blood plasma and cerebrospinal fluid (CSF). Viral genetic populations in blood plasma and cell-free CSF were compared by V1/V2 env heteroduplex tracking assay (HTA). An asterisk indicates necropsy specimens. All HTA lanes shown were confirmed for efficient and reproducible viral RNA template sampling. Lanes marked by an X indicate specimens for which viral RNA template concentrations were inadequate for efficient reverse-transcription polymerase chain reaction (RT-PCR) amplification or HTA reproducibility. Charts indicate the level of HTA band pattern discordance between blood and CSF viral populations at each time point, taking into account the presence of unique variants in one compartment or another as well as differences in the relative abundance of shared variants. To account for minor differences in band-abundance reproducibility, all values reported for percentage of blood/CSF discordance were corrected for the average percentage of discordance between individual RT-PCR and HTA replicates of blood and CSF for each time point. The average percentage of discordance values for replicates at all time points were 13.3%, 10.4%, and 9.25% for macaques D110, D081, and C002, respectively. Each data point shown represents the average corrected percentage of discordance of blood and CSF HTA patterns for each time point, with error bars indicating SDs.

Comparison of blood and CSF viral populations. We next compared SIVsm genetic structure in blood and CSF. Viral env V1/V2 populations in both compartments were analyzed by HTA. The level of blood plasma and CSF viral population discordance, which accounts both for the presence of unique variants in one compartment versus another and for differences in the relative abundance of variants shared between both compartments, was then quantified.

We observed 2 distinct patterns of blood/CSF viral evolution in the macaques (figure 3). In macaques D110 and D081, blood and CSF V1/V2 populations were nearly indistinguishable over the full course of infection. Even when major changes occurred
in the blood viral population (e.g., for D110 at days 86–99), the viral population in CSF mirrored those changes. Only 1 late sampling time point for D110 displayed significant discordance between blood and CSF, which reflected a change in the relative abundance for a major genetic variant shared between blood and CSF. These results suggest that blood and CSF viral genetic populations can be well equilibrated throughout the full course of infection.

In contrast to the observation of blood/CSF viral genetic concordance in macaques D110 and D081, we observed a discordant pattern between blood and CSF viral populations in macaque C002. The major V1/V2 populations were shared between the blood and CSF compartments at day 14, but by day 27 a major independent variant had emerged in CSF. After a period of little or no detectable virus in CSF, the CSF viral populations reemerged and remained relatively discordant from those in blood for the remainder of the infection. Over all sampling time points, the level of blood/CSF viral genetic discordance was >3-fold greater in C002 than in D110 and D081 (figure 4). These results suggest that a significant fraction of the CSF viral population in C002, but not in D110 or D081, was evolving independently from that in the periphery and was likely compartmentalized to the CNS.

**Biological markers for virus-associated CNS inflammation.** Although 3 macaques is not a sufficient sample size to fully determine the relationship between viral CSF compartmentalization and CNS disease, we hypothesized that the 2 distinct patterns of blood/CSF viral population dynamics may reflect differences in SIV-associated neurological involvement. The proinflammatory chemokine MCP-1 plays an important role in the trafficking of inflammatory cells in the CNS, and elevated levels of MCP-1 in CSF are associated with both HIV- and SIV-associated neurological disease [36–39]. We quantified MCP-1 levels in prechallenge and necropsy CSF specimens by ELISA. Although there were no patterns with respect to prechallenge CSF MCP-1 levels and neurological disease outcome in the different animals, macaque C002 had the highest level of CSF MCP-1 at end stage (figure 5A). Furthermore, there was an ~2-fold higher level of CSF MCP-1 at the end-stage versus prechallenge time points for C002, whereas macaques D110, D081, and C123 (uninfected) had unchanged or reduced levels of CSF MCP-1 at the time of euthanasia or killing (figure 5B).
The trafficking of monocytes and macrophages into the brain is also a common feature of SIV- and HIV-associated neurological disease [2, 39–42]. Paraffin-embedded sections of the caudate nucleus and overlying frontal cortex were stained with an antibody to CD68, which specifically stains macrophages. Stained cells were counted in the cortex, subcortical white matter, and caudate. Little or no significant staining was observed in the brains of macaques D110, D081, or C123 for all regions examined (figure 6). In contrast, we detected a moderate number of CD68+ cells in perivascular regions of the brain of macaque C002. The most intense staining was localized to the caudate and subcortical white matter, regions of the brain often afflicted in human patients with HIV-associated dementia [43, 44]. Therefore, the detection of blood/CSF viral genetic discordance in C002 was accompanied by elevated CSF MCP-1 levels and infiltration of CD68+ macrophages into the brain.

**DISCUSSION**

This report describes the first comprehensive longitudinal analysis of complex SIVsm genetic populations in blood and CSF throughout the course of infection. At the first sampling time point (day 14 or 15 after challenge), no CSF-specific viral variants were detected in any of the infected macaques, although differences in the relative abundance of blood/CSF-shared genetic variants were already apparent at this early time point in macaque C002. The presence of a complex, equilibrated viral population in CSF this early during infection, especially notable...
in macaques D110 and D081, suggests that the CSF compartment is readily accessible to most, if not all, SIVsm variants replicating in the periphery.

We observed 2 strikingly different patterns of viral population dynamics between blood and CSF throughout the remainder of infection, one in which blood and CSF viral populations were concordant (D110 and D081) and another in which they were discordant (C002). At least 2 possible mechanisms can explain the concordant patterns in macaques D110 and D081. One is that SIVsm present in CSF was replicating locally in the CNS, but selective pressures in the CNS and periphery targeting the V1/V2 region of Env were identical at all stages of infection. However, it seems unlikely that selective pressures targeting Env are identical in the 2 compartments throughout infection. The more likely explanation is that SIVsm was not replicating locally in the CNS in D110 or D081. In this case, virus and/or virus-infected cells were trafficking between the periphery and CSF throughout all stages of infection, with no selective barrier between the 2 compartments.

The discordant pattern of blood and CSF viral populations in macaque C002 suggests that at least a portion of the complex viral population in CSF had undergone a distinct evolutionary pathway that permitted enhanced replication in the CNS. Notably, discordance of blood and CSF viral populations was apparent within 1 month after challenge, before the occurrence of major genetic changes in the blood viral population and also before the development of a strong SIVsm H4 neutralizing antibody response (data not shown) [25]. This observation suggests that selective forces other than neutralizing antibody were differentially acting on viruses produced locally within the CNS of this macaque, at least at this early time point. We speculate that the CSF compartmentalization of viral populations in this macaque reflected adaptation of the virus to replicate in cells of the CNS, with the CNS-unique selective forces on Env predominantly occurring at cell entry. Alternatively, the establishment of a genetically distinct virus replicating in the CNS may have involved a founder effect, although these explanations are not mutually exclusive.

In cross-sectional studies of infected human volunteers, we have observed a strong association between HIV-1 compartmentalization in CSF and neurological disease [11, 20] (P.R.H. and R.S., unpublished data). We have detected little CSF compartmentalization of HIV-1 during primary infection, variable CSF compartmentalization in subjects with minor forms of neurological impairment, and high levels of CSF compartmentalization in subjects with HIV-associated dementia. Others have observed both genotypic and phenotypic compartmentalization of HIV-1 env between brain and lymphoid tissues [18, 45–49]. A recent study using an accelerated SIV/macaque neuropathogenesis model demonstrated that a neurovirulent variant of SIV was present in similar proportions relative to other coexisting genetic variants in both peripheral blood mononuclear cells and brain tissue early during infection but became more enriched in the brain as disease progressed [50].

On the basis of these previous observations, we hypothesized that the different patterns of blood/CSF concordance we observed in macaques were similarly associated with different levels of neurological disease. Our preliminary characterization of 2 disease markers, CSF MCP-1 and brain infiltration of CD68+ cells, revealed CNS inflammation in macaque C002 but not in macaques D110 or D081. Therefore, the discordance of SIVsm populations in blood and CSF was associated with markers of neurological disease, although we are cautious in this conclusion because we characterized only 3 infected animals in the present study. Additional experimental infections are under way to characterize more fully these and other neurological disease markers throughout infection so as to address more systematically the relationship between viral CSF compartmentalization and neurological disease.

In conclusion, the present study illustrates the complex relationships between SIVsm populations in multiple compartments throughout the course of infection. We revealed that at least 2 different patterns of blood/CSF SIVsm population dynamics can be observed throughout infection. Our results demonstrate that SIVsm populations can frequently traffic between blood and CSF without selection for any particular genotypes, although in some cases viral populations can also evolve independently in the 2 compartments. Further study of the role played by viral CSF compartmentalization in SIVsm-associated neurological disease is warranted, and we anticipate that the SIVsm E660 model and methods described in this report will complement other approaches to study the mechanisms of SIV and HIV neuropathogenesis.

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