Identification and Characterization of HIV-1

Latent Viral Reservoirs In Peripheral Blood

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Abstract

Plasma viral load and CD4 counts are effective for clinical monitoring, but they do not give a full representation of HIV-1 quasispecies in cellular reservoirs, the major repository of replication-competent HIV-1 in infected individuals. We sought to develop a diagnostic system that could stimulate the replication-competent HIV-1 reservoirs for enhanced clinical monitoring including selection of antiretroviral regimens. Whole blood from 45 HIV-infected individuals was collected into 1 ViraStim™ HIV-1 activation tube and 1 EDTA tube. Samples were tested for viral load and cell-type specific HIV-1 replication. Further, 7 matched activated/non-activated samples were sequenced using the TRUGENE® HIV-1 Genotyping Kit. The percentage of patients with replication-competent virus in PBMCs varied depending on the baseline plasma viral load in the EDTA tubes. Six out of 24 patients with starting plasma viral load <20 copies/mL (cp/mL), 6 out of 8 patients with starting viral loads >20 and <1000 cp/mL, and 8 patients out of 13 with starting viral loads >1000 all showed an increase in viral replication greater than 5-fold. This increase came from cellular reservoirs in blood as determined by Simultaneous Ultrasensitive Subpopulation Staining/Hybridization In Situ (SUSHI). When comparing resistance genotypes in plasma from activation tubes compared to EDTA tubes for 7 patients, all patients showed additional mutations in the activation tube, while 3 patients demonstrated additional genotypic resistance determinants compared to EDTA tubes. We show that HIV-1 viral replication can be stimulated directly from infected whole blood.
Introduction

The hallmark of antiretroviral drug monitoring in HIV-1-infected individuals has been plasma viral load and CD4 counts. Newer technologies have been developed to elucidate the cellular reservoirs of HIV-1 actively producing virus at the time of blood draw; however, these technologies provide little information on latent reservoirs containing replication competent HIV-1. In peripheral blood, a significant proportion of peripheral blood mononuclear cells (PBMCs) contain HIV-1 DNA (1, 2), though very few HIV-1 DNA-positive PBMCs can be reactivated to express viral mRNA, implying that only a small fraction of cells in the peripheral blood are transcriptionally active and considered “active reservoirs.”

The utilization of combination antiretroviral therapy (ART) for HIV-1 infection has generated interest in mechanisms by which the virus can persist in the body despite the presence of drugs that are designed to inhibit key steps in the virus life cycle including infection of new cells. Viral reservoirs established early in infection represent a major obstacle to the efficacy of antiretroviral drugs currently in use and will be a significant consideration in efforts to develop a treatment approach for cure of HIV-1 infection (3). Because PBMCs and tissues such as lymph nodes respond with similar decay kinetics during ART, PBMCs might be an important surrogate for HIV analysis in lymphoid tissue reservoirs (4).
Recently, commercial laboratories have begun developing tests designed to detect and/or quantify cell-associated (CA), integrated HIV proviral DNA, as well as unintegrated (episomal) HIV DNA (5). These assays are PCR-, nested-PCR, or alu-PCR in the case of integrated HIV-1 DNA based and are performed on either whole blood or ficoll-separated peripheral blood mononuclear cells (PBMCS) (5, 6). Though useful to estimate the total viral burden (HIV-1 DNA) in individuals, these assays lack the ability to determine the replication competence of the HIV-1 DNA residing in cells. Further, plasma may not be the best source of virus for antiretroviral resistance testing, as plasma virus consists of defective, non-replication-competent virus in addition to replication-competent HIV (7, 8). In addition, studies have shown that different reservoirs of HIV in an individual may exhibit different genotypic resistance determinants (9, 10). These findings could significantly alter the choice of antiretroviral drugs used for antiretroviral therapy in HIV-1-infected individuals and allow for the possibility of eradication strategies that focus on inducing the replication-competent, latently infected cells to produce virus. To that end, we sought to develop a diagnostic system that could quantify and characterize virus produced from the replication-competent HIV-1 reservoir and permit antiretroviral resistance testing to be performed on a broader representation of an HIV-infected individual's quasispecies.
Materials and Methods

Study Subjects
Forty-five HIV-1-positive patients from multiple sites (BioCollections Worldwide, Inc. Miami, FL) were recruited. Patients were required to have had a CD4 count and HIV-1 viral load test performed within the last 6 months. CD4 counts, HIV-1 viral load, current ART therapy, gender, date of birth, ethnicity, country and state of origin, and race were recorded. Following IRB-approval and consent, patients were required to be able to give 32 mL of whole blood. Whole blood collection requirements were >2 mL to ensure adequate plasma for viral load testing.

Blood collection
For each subject, whole blood was collected into one of three HIV-1 activation tubes (ViraStim™ tubes, IncellDx, Menlo Park, CA), and one EDTA tube. After collection, the tubes were shaken vigorously for 5 seconds to ensure that the entire inner surface of the tube had been coated with the blood. The EDTA tube was centrifuged for plasma collection by spinning the tube at 2500 RCF (g) for 30 minutes at room temperature (15 - 30°C), upon receipt of the blood collection tube, and within 24 hours of collection. Upon receipt of the three HIV-1 activation tubes, the tubes were mixed again, prior to incubation, as described above. The ViraStim™ tubes were transferred to a 37°C incubator as soon as possible, and within 24 hours of collection. Tubes were incubated for 48 hours ± 2 hours. After incubation of the tubes at 37°C, plasma was collected by centrifuging tubes for 30 minutes at 2500 RCF (g), at room temperature (15 -
Plasma samples were collected, stored at \(-20^\circ\text{C}\) (for up to 2 weeks) and the viral load in the plasma was measured using the Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test.

The protocol has been previously described in general for similar applications (11-13). Known significant cellular reservoirs (3, 14) were selected for these analyses, and further delineated into memory and naïve T-cells, in resting and activated states, and monocyte to macrophage differentiation to discern relative levels of gag-pol+ reservoirs. The HIV-1 probe cocktail contains oligos covering 90% conserved regions across HIV-1 subtypes A, B, C, AE, AG, and BF (ViroTectVR, IncellDx Inc, Menlo Park, CA). Fresh whole blood was transferred to a Falcon tube (Becton, Dickinson, Bedford, MA,) then 1 mL of Reagent 1 (one-step fixation/permeabilization, IncellDx, Inc, Menlo Park, CA) was added to the tubes followed by incubation at 43°C for 30 minutes to lyse the red cells. An appropriate dilution of antibodies (BDIS, San Jose, CA), in predetermined combinations was added to the cell suspension and incubated at room
temperature for 30 minutes. Following antibody hybridization, cells were resuspended in 1 mL Reagent 2 (prehybridization buffer 1) inverted gently, and centrifuged. The supernatant was aspirated. This was repeated with Reagent 3 (prehybridization buffer 2). The supernatant was aspirated followed by the addition of Reagent 4 (hybridization buffer) and Reagent 5 and incubated in a 43°C bath for 30 minutes. Following incubation, 1 mL of prewarmed (43°C) Reagent 6 (Stringency Wash 1) was added, the tube inverted gently and centrifuged. The supernatant was aspirated, the cell pellet gently resuspended in the residual fluid followed by the addition of 1 mL prewarmed (43°C) Reagent 7 (Stringency Wash 2) and incubated in a 43°C water bath for 15 minutes. The tube was centrifuged, the supernatant aspirated, the cell pellet gently resuspended in the residual fluid followed by the addition of PBS. Samples were then collected and analyzed on the EC800 flow cytometer (Sony Biotechnology, Champaign, IL).

HIV+ and HIV- Controls.

The ACH-2 cell line containing a single copy of integrated HIV-1 proviral DNA per cell with limited to no expression of HIV-1 mRNA is routinely used as a control (12, 15), and residual HIV-1 mRNA is not detected with the SUSHI gag-pol probes (below level of detection) (12). Induction of ACH-2 HIV-1 RNA expression with phorbol 12-myristate 13-acetate (PMA) at 80 µg/mL PMA (Sigma Aldrich, St. Louis, MI) was used as a positive control to verify hybridization and signal detection of SUSHI gag-pol probes (12, 15). This cell line was used as a positive (stimulated) and an operationally negative (unstimulated) control in the
first publication describing the SUSHI technology (12), which demonstrated a linear response between the percentage of stimulated ACH-2 cells as measured by the SUSHI fluorescent HIV-1 gag-pol probes versus actual percentage of stimulated ACH-2 cells by dilution.

**HIV-1 Reservoir sequencing**

Plasma samples from the EDTA and ViraStim tubes were collected, stored at ≤ -20°C (for up to 2 weeks) and then genotyped using the TRUGENE® HIV-1 Genotyping Kit which confers resistance to specific types of antiretroviral drugs. It is indicated for use in monitoring and treating HIV infection.

**Statistical Analysis**

The correlation between fold changes in pVL and fold changes in cell type specific viral replication were determined using SigmaPlot 12.5 software. For pVL samples <20, a copy number of 2 was used as it represents the analytic sensitivity of the assay. Fold changes in replication were determined using the percentage of infected cells in the CD3+, CD4+ cell population. A correlation above 0.6 represents a strong correlation between the two groups of measurements. P-values <0.05 were considered statistically significant.
Results

Quantification of replication-competent HIV-1

To determine the relative amounts of replication competent HIV-1 in hematopoietic cells in peripheral blood, we used specialized blood collection tubes containing HIV-1 inducing compounds (ViraStim™ tubes). By collecting blood plasma from EDTA tubes as a control and ViraStim tubes, plasma viral load differences were quantified. The difference between plasma viral load in EDTA tubes and plasma viral load in ViraStim tubes incubated for 48 hours at 37°C represents the amount of replication competent viral residing in cells contained within the blood sample. As demonstrated in Figure 1A-C, replication competent virus was induced to replicate independent of the plasma viral load. The percentage of patients with replication competent virus in PBMCS varies depending on the baseline plasma viral load in the EDTA tubes. Six out of 24 patients with starting plasma viral load <20 copies/mL (cp/mL), 6 out of 8 patients with starting viral loads >20 and <1000 cp/mL, and 8 patients out of 13 with starting viral loads >1000 all showed an increase greater than 5-fold (0.5 log).

Identification of replication competent HIV-1 reservoirs

To confirm the results seen in viral activation tubes and to identify the reservoirs of HIV-1 in PBMCS harboring replication competent virus, we performed SUSHI on cells in paired EDTA and ViraStim tubes and compared the percentage of cells with replicating HIV-1 between the two tubes. In the absence of replication stimulation in the EDTA tubes, the range of transcriptionally active CD3+, CD4+
cells was 0% to 0.94%. Cells collected and stimulated in ViraStim™ tubes demonstrated increased HIV-1 replication ranging from 1.68% to 15.16% of CD3+, CD4+ cells (Figure 2A). Hybridization performance was confirmed in this set of experiments by use of stimulated and unstimulated ACH-2 cells (Figure 2B).

Increased HIV-1 replication in CD3+, CD4+ T-lymphocytes as determined by SUSHI suggests that the source of increased virus seen in the viral activation tubes may come from activated T-cells. In particular, the extent of plasma viral load fold-increases was proportional to the fold-increase in cells containing replicating virus (Figure 3, r²=0.7 P=0.006).

Assessment of HIV-1 quasispecies specific genotypic resistance patterns

Because HIV-1 virus contained in plasma represents only a single viral reservoir in HIV-1 infected individuals, and, because this reservoir is the only reservoir routinely assessed during anti-retroviral resistance testing, we used plasma virus collected in ViraStim tubes to compare resistance phenotypes in plasma to resistance phenotypes in virus derived from PBMCS and plasma. As shown in Table 1, 3 out of 7 patients demonstrated additional genotypic resistance determinants in ViraStim tubes compared to EDTA tubes. When comparing resistance genotypes in plasma from activation tubes compared to EDTA tubes for 7 patients, all patients showed additional mutations in the activation tube, while 3 patients demonstrated additional genotypic resistance determinants compared to EDTA tubes.
Discussion

With HIV eradication becoming an emerging goal of HIV medicine, elucidation and monitoring of HIV-1-infected reservoirs is critical (16). To that end, we performed studies aimed at combining analyses of replication-competent HIV-1 reservoirs with the virus already present in plasma to obtain a broader representation of HIV-1 quasispecies.

ViraStim™ stimulation on whole blood resulted in a 5-fold (0.5 log) increase in plasma viral load in 6 out of 24 patients with starting plasma viral load <20 cp/mL, 6 out of 8 patients with starting viral loads >20 and <1000 cp/mL, and 8 patients out of 13 with starting viral loads >1000 compared to viral load measured in EDTA tubes. The cutoff of 5-fold (0.5 log) was chosen because it is greater than what would be expected from the intra-run variability of viral load assays. Though many approaches and several studies have demonstrated the ability to stimulate viral replication from PBMCS in *in vitro* cultures (17, 18), this is the first report of a rapid, clinical approach to stimulate viral replication in a clinical sample. The percentage of patients with pVL <20 cp/mL was expected to be low but not zero based on previous publications quantifying cell associated unspliced and spliced HIV-1 RNA in patients on suppressive anti-retroviral therapy (19). The percentages in the other two non-suppressed groups was variable which is not unexpected based on the fact that these patients may have different numbers of HIV-1 DNA+, replication competent cells and patient to patient variability of free virus clearance (20).
To further demonstrate that the increase in viral production was a result of stimulating latent, replication-competent virus, we performed SUSHI analysis on HIV-1 reservoirs (e.g., CD3 and CD4 T-cells). We demonstrated a correlation between the fold increase in HIV replication in intact cells and the fold increase in the ViraStim™ tubes relative to the EDTA tubes. We have previously shown the utility of SUSHI in measuring decreases in viral replication associated with antiretroviral therapy (21, 22). In the present study, we demonstrate utility in the detection of increased viral replication in HIV-1 reservoirs infected with replication competent virus; a potentially useful tool in the monitoring of eradication strategies.

As additional evidence that the increase in viral replication seen in ViraStim tubes reflects a broader quasispecies representation than in the sample tube, we found differences in resistance mutations and amino acid changes when compared to virus from an EDTA control tube. This suggests that the viral phenotypes detected by sequencing out of plasma are not fully representative of the viral quasispecies in cellular reservoirs as suggested by other studies using alternate methodologies (23). Plasma contains a mixture of both replication-competent and replication-defective virus. HIV phenotyping is typically performed on the mixture of the two cloned into replication-competent backgrounds providing a resistance profile that is skewed by cloning of nonfunctional virus particles. In addition, virus that is latent and currently unexpressed is not represented in normal plasma: “It has been shown that a cessation of ART treatment or a switch in antiretroviral
drugs in patients treated for more than 2 years with suboptimal drug regimens result in a replacement of the resistant virus in the plasma by wild-type variants. In the majority of the patients studied, the replacement of the mutant by the wild-type virus was abrupt and fast, indicating that it was the result of the reappearance of archived wild-type virus and not of the reversal of mutations in the resistant variants. If wild-type virus persists in the latent reservoir for such a long time, then it could be postulated that drug-resistant strains too will be conserved.” (24) However, plasma-based measurements on isolated nucleic acids yield no information on the cell types and subpopulations that are productively infected and, thus, are contributing to the free virus pool in the plasma (11). To investigate the source of the increase in virus and the change in resistance profiles described in the present study, we looked at the cellular reservoirs and measured mRNA expression of lymphocyte subsets. As utilized in previous reports from this laboratory (22), we used CD3+, CD4+ gating rather than light scatter gating for lymphocytes since we previously demonstrated that HIV-1 infected lymphocytes are much larger than typical uninfected lymphocytes and can fall outside of a normal lymphocyte gate (22). Since replication competent CA HIV-1 reservoirs stand in opposition to HIV eradication, new strategies have been employed to reactivate these reservoirs using compounds such as histone deacetylase inhibitors (HDACs) among others (16). The approach presented in this paper could be used to prescreen patients who would respond best to certain ART regimens, given that the ViraStim tubes demonstrate the degree of stimulation to be expected at least in the PBMC reservoir, which, as previously mentioned,
reflects the total tissue reservoir of HIV. Further the ART backbone used in patients induced by HDAC could be tailored based on sequencing data on the induced PBMC reservoir as described in the present study.

Conclusion

This study described here demonstrates that HIV-1 viral replication can be stimulated using ViraStim™ stimulation of whole blood. The sequencing results showed that 3 cases had additional drug resistance following stimulation. The technique described here has the potential to more accurately identify antiretroviral resistance and to inform treatment regimens in HIV-infected patients.

Acknowledgment

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Conflict of Interest

All authors are employees of IncellDx, Inc the manufacturer of the ViraStim™ tubes.
References


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**Figures and Tables**

**Figure 1.** Plasma viral load comparison between EDTA tubes and ViraStim™ tubes. Six out of 24 patients with starting plasma viral load (A)<20 copies/mL, (B) 6 out of 8 patients with starting viral loads >20 and <1000 cp/mL, and (C) 8 patients out of 13 with starting viral loads >1000 demonstrated increases >0.5 logs in the ViraStim™ compared to the EDTA tubes.

**Figure 2.** (A) Flow cytometric histograms demonstrating HIV-1 replication in CD3+, CD4+ T-lymphocytes using SUSHI. PBMCS from ViraStim™ tubes showed increased replication relative to PBMCs from EDTA tubes. (B) Histogram overlay of HIV-1 hybridization controls using PMA-stimulated ACH-2 cells (red) that express high levels of HIV-1 mRNA as a positive control and unstimulated ACH-2 cells (black) which express little if any HIV-1 mRNA as a negative control.

**Figure 3.** Fold changes in HIV-1 replication in the CD3+, CD4+ reservoir and in plasma viral load using the formula ViraStim™ tube/EDTA tube. Included in the subset of patients were samples with adequate cells to determine the replication in cellular reservoirs. Increases of viral replication in cells (x-axis) correlated ($r^2$-0.7, $P=0.006$) with increases of virus in plasma (y-axis).
Initial VL < 20 cp/mL

VL (x 10^3 cp/mL)

EDTA
ViraStim™

Patient ID#
Figure 3

Plasma viral load (fold change) vs. CD3+, CD4+, HIV-1 mRNA+ cell reservoir (fold change)
Table 1. Sequencing comparison between plasma derived from EDTA tubes and ViraStim™ tubes. Plasma from ViraStim™ tubes showed additional mutations including mutations that conferred anti-retroviral resistance in 3 out of 7 individuals (grey).

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Tipranavir + Ritonavir (TPV/r) Possible Resistance
Rilpivirine (RPV) Resistance
Atazanavir (ATV) Resistance
Stavudine (d4T) Possible Resistance
Tenofovir (TDF) Possible Resistance
Darunavir + Ritonavir (DRV/r) Possible Resistance
Fosamprenavir (FPV) Resistance
Indinavir (IDV) Resistance
Lopinavir + Ritonavir (LPV/r) Possible Resistance
Nelfinavir (NFV) Possible Resistance
Saquinavir + Ritonavir (SQV/r) Possible Resistance

Table 1. Sequencing comparison between plasma derived from EDTA tubes and ViraStim™ tubes. Plasma from ViraStim™ tubes showed additional mutations including mutations that conferred anti-retroviral resistance in 3 out of 7 individuals (grey).