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Identification and Characterization of HIV-1 Latent Viral Reservoirs In Peripheral Blood

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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 might stimulate the replication-competent HIV-1 reservoirs for enhanced clinical monitoring, including selection of antiretroviral regimens. Whole-blood samples from 45 HIV-infected individuals were 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/nonactivated samples were sequenced using the Trugene HIV-1 genotyping kit. The percentage of patients with replication-competent virus in peripheral blood mononuclear cells (PBMCs) varied, depending on the baseline plasma viral load in the EDTA tubes. Six out of 24 patients with a starting plasma viral load of <20 copies/ml (cp/ml), 6 out of 8 patients with starting viral loads of >20 and <1,000 cp/ml, and 8 out of 13 patients with starting viral loads of >1,000 all showed increases in viral replication of >5-fold. These increases came from cellular reservoirs in blood as determined by simultaneous ultrasensitive subpopulation staining/hybridization in situ (SUSHI). When resistance genotypes in plasma from activation tubes were compared to those from EDTA tubes for 7 patients, all patients showed additional mutations in the activation tube, while 3 patients demonstrated additional genotypic resistance determinants. We show that HIV-1 viral replication can be stimulated directly from infected whole blood. The sequencing results showed that 3 of 7 cases demonstrated additional drug resistance following stimulation.

The hallmark of antiretroviral drug monitoring in HIV-1-infected individuals has been plasma viral load (pVL) 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), although 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 curing 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-based in the case of integrated HIV-1 DNA and are performed on either whole-blood or Ficoll-separated PBMCs (5, 6). Although useful for estimating the total viral burden (HIV-1 DNA) in individuals, these assays lack the ability for determining 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 might 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 quantifies and characterizes virus produced from the replication-competent HIV-1 reservoir and permits 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, gender, date of birth, ethnicity, country and state of origin, and race

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were recorded. Following institutional review board (IRB) approval and consent, patients were required to be able to give 32 ml of whole blood. The whole-blood collection requirement was >2 ml to ensure adequate plasma for viral load testing.

Blood collection. For each subject, whole-blood samples were collected into three 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 s to ensure that the entire inner surface of the tube had been coated with the blood.

The EDTA blood collection tube was centrifuged for plasma collection by spinning at 2,500 relative centrifugal force (RCF) (*g*) for 30 min at room temperature (15 to 30°C), upon receipt and within 24 h of collection. Upon receipt, the three HIV-1 activation 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 h of collection. Tubes were incubated for 48 h ± 2 h. After incubation of the tubes at 37°C, plasma was collected by centrifuging the tubes for 30 min at 2,500 RCF (*g*), at room temperature (15 to 30°C). Plasma samples were collected and stored at −20°C or lower (for up to 2 weeks), and the viral load in the plasma was measured using the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test.

Plasma samples were collected and stored at −20°C or lower (for up to 2 weeks), and the viral load in the plasma was measured using the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test with a lower limit of detection of <20 copies/ml of plasma.

HIV-1 reservoir quantification using SUSHI. The simultaneous ultrasensitive subpopulation staining/hybridization in situ (SUSHI) protocol was 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 naive T cells in resting and activated states, and monocyte to macrophage differentiation was used to discern relative levels of gag-pol$^+$ reservoirs. The HIV-1 probe cocktail contains oligonucleotides covering 90% conserved regions across HIV-1 subtypes A, B, C, AE, AG, and BF (ViroTect; IncellDx Inc., Menlo Park, CA). Fresh whole blood was transferred to a Falcon tube (Becton Dickinson, Bedford, MA), and then 1 ml of reagent 1 (one-step fixation/permeabilization; IncellDx, Inc.) was added to the tubes followed by incubation at 43°C for 30 min to lyse the red cells. An appropriate dilution of antibodies (BD Immunocytometry Systems, San Jose, CA), in predetermined combinations, was added to the cell suspension and incubated at room temperature for 30 min. Following antibody hybridization, cells were resuspended in 1 ml reagent 2 (prehybridization buffer 1), and the tube was 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 (HIV mRNA probe) and incubated in a 43°C bath for 30 min. Following incubation, 1 ml of prewarmed (43°C) reagent 6 (stringency wash 1) was added, and the tube was inverted gently and centrifuged. The supernatant was aspirated, and the cell pellet was 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 min. The tube was centrifuged, the supernatant was aspirated, and the cell pellet was gently resuspended in the residual fluid followed by the addition of phosphate-buffered saline (PBS). Samples were then collected and analyzed on the EC800 flow cytometer (Sony Biotechnology, Champaign, IL).

HIV-positive and -negative 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 the 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, MO) 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 the actual percentage of stimulated ACH-2 cells by dilution.

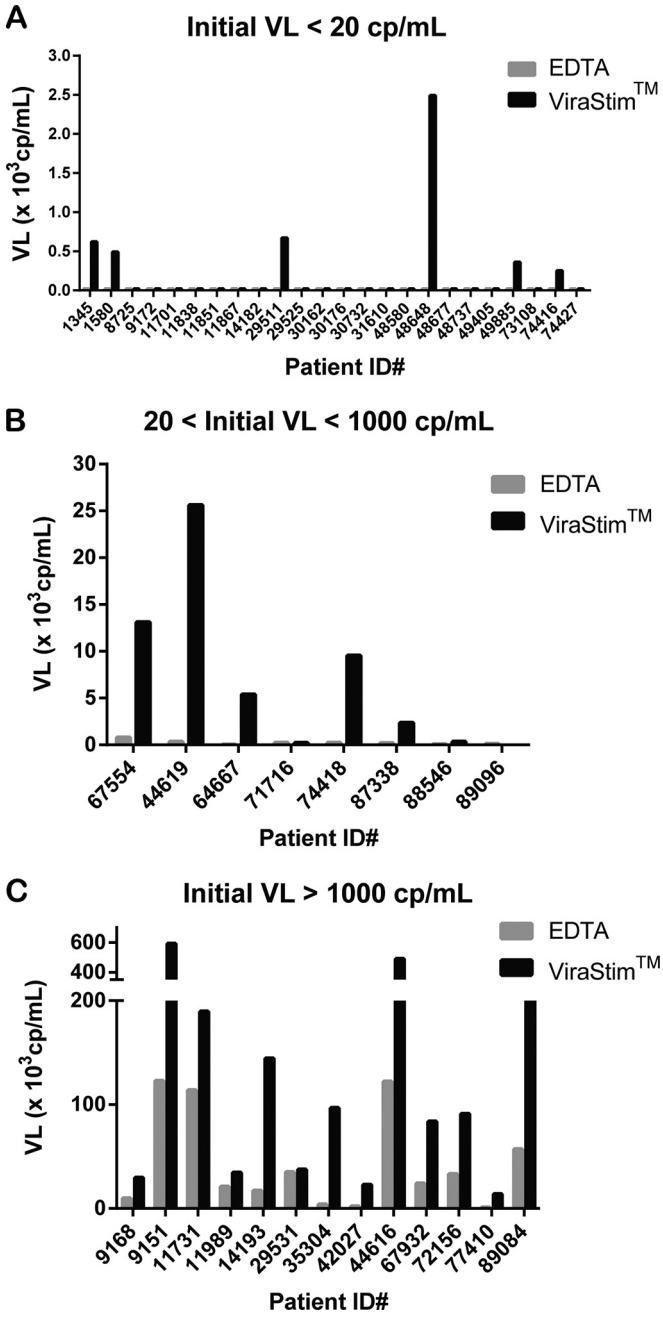
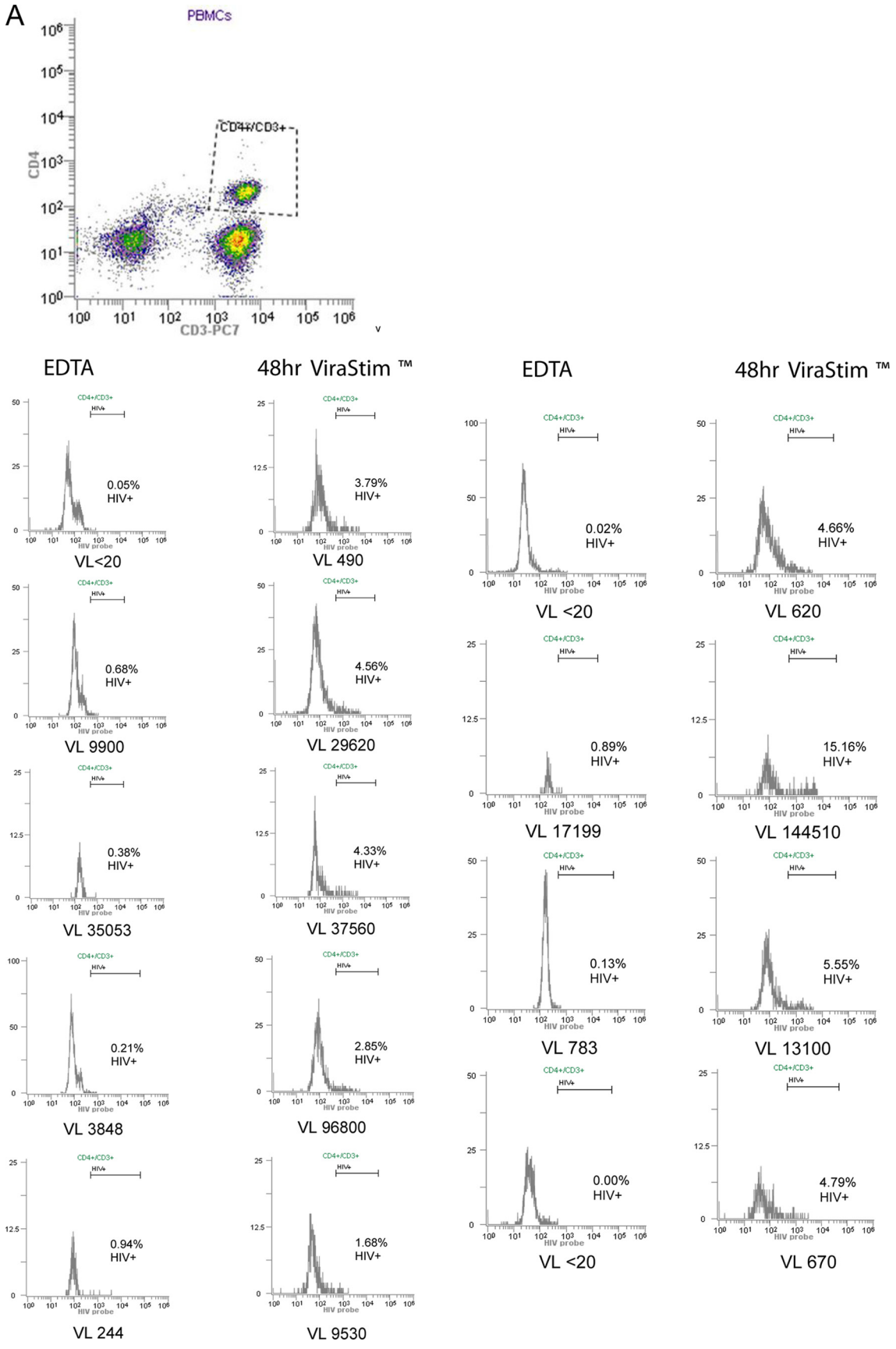


FIG 1 Plasma viral load (VL) comparison between EDTA tubes and ViraStim tubes. Six out of 24 patients with starting plasma viral loads of <20 copies/ml (cp/ml) (A), 6 out of 8 patients with starting viral loads of >20 and <1,000 cp/ml (B), and 8 out of 13 patients (C) with starting viral loads of >1,000 demonstrated increases of >0.5 log in the ViraStim tubes compared to the EDTA tubes.

HIV-1 reservoir sequencing. Plasma samples from the EDTA and ViraStim tubes were collected, stored at −20°C or lower (for up to 2 weeks), and then genotyped using the Trugene HIV-1 genotyping kit,



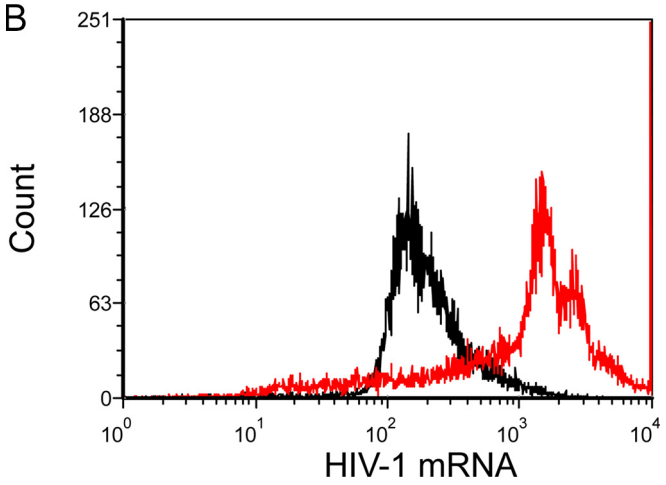


FIG 2 (A) Flow cytometric histograms demonstrating HIV-1 replication in $CD3^+$ and $CD4^+$ T lymphocytes using SUSHI. PBMCs from ViraStim tubes showed increased replication relative to that of PBMCs from EDTA tubes. (B) Histogram overlay of the HIV-1 hybridization controls using PMA-stimulated ACH-2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

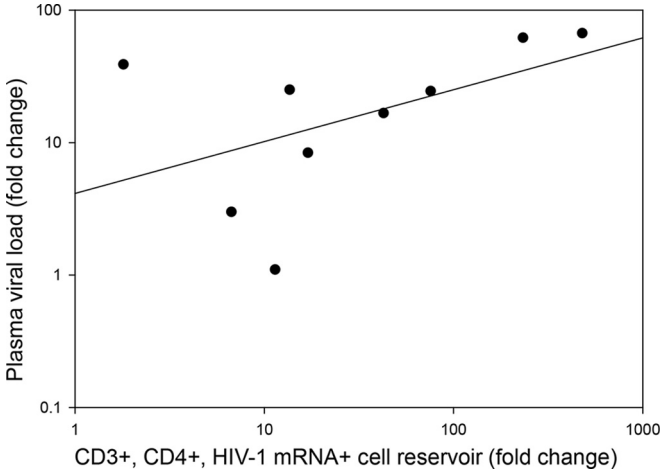


FIG 3 Fold changes in HIV-1 replication in the $CD3^+$ $CD4^+$ reservoir and in the plasma viral load using the formula ViraStim tube/EDTA tube. Included in the subset of patients were samples with adequate cells to determine the replication in cellular reservoirs. Increases in viral replication in cells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

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 of <20 copies/ml, 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 of >0.6 represents a strong correlation between the two groups of measurements. P values of <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 from ViraStim tubes, plasma viral load differences were quantified. The difference between plasma viral loads in EDTA tubes and plasma viral loads in ViraStim tubes incubated for 48 h at 37°C represents the amount of replication-competent virus residing in cells contained within the blood sample. As demonstrated in Fig. 1A to C, replication-competent virus was induced to replicate independently 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 a starting plasma viral load of <20 cp/ml, 6 out of 8 patients with starting viral loads of >20 and <1,000 cp/ml, and 8 out of 13 patients with starting viral loads of >1,000 all showed increases of >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 percentages 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 (Fig. 2A). Hybridization performance was confirmed in this set of experiments by use of stimulated and unstimulated ACH-2 cells (Fig. 2B). Increased HIV-1 replication in $CD3^+$ $CD4^+$ T lymphocytes as determined by SUSHI suggests that the 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 ($r^2 = 0.7$, $P = 0.006$) (Fig. 3).

Assessment of HIV-1 quasispecies-specific genotypic resistance patterns. Because HIV-1 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 antiretroviral 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 resistance genotypes in plasma from activation tubes were compared to those in EDTA tubes for 7 patients, all patients showed additional mutations in the activation tube, while 3 patients demonstrated additional genotypic resistance determinants compared to those in 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 to combine 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 of whole-blood samples resulted in a 5-fold (0.5-log) increase in plasma viral loads in 6 out of 24 patients with starting plasma viral loads of <20 cp/ml, 6 out of 8

TABLE 1 Sequencing comparison between plasma derived from EDTA tubes and ViraStim tubes

Patient	Results for plasma from EDTA tubes		Results for plasma from ViraStim		No. of mutations detected in ViraStim not found in EDTA
	Drug class[a]	Resistance interpretation	Drug class[a]	Resistance interpretation	
4318		No evidence of resistance		No evidence of resistance	
70148	d4T	Possible resistance	d4T	Possible resistance	
	TDF	Possible resistance	TDF	Possible resistance	
	AZT	Resistance	AZT	Resistance	
	ABC	Possible resistance	ABC	Possible resistance	
	ddI	Resistance	ddI	Resistance	
	ATV	Resistance	ATV	Resistance	
	ATV/R[b]	Resistance	ATV/R[b]	Resistance	
	DRV/R	Possible resistance	DRV/R	Possible resistance	22
	FPV	Resistance	FPV	Resistance	
	FPV/R[b]	Resistance	FPV/R[b]	Resistance	
	IDV	Resistance	IDV	Resistance	
	IDV/R[b]	Possible resistance	IDV/R[b]	Possible resistance	
	LPV/R	Possible resistance	LPV/R	Possible resistance	
	NFV	Possible resistance	NFV	Possible resistance	
	SQV/R	Possible resistance	SQV/R	Possible resistance	
			TPV/R[c]	Possible resistance	
11989	ABC	Resistance	ABC	Resistance	
	ddI	Resistance	ddI	Resistance	
	3TC/FTC	Resistance	3TC/FTC	Resistance	4
	EFV	Resistance	EFV	Resistance	
	ETR	Possible resistance	ETR	Possible resistance	
	NVP	Resistance	NVP	Resistance	
72093	3TC/FTC	Resistance	3TC/FTC	Resistance	
	EFV	Resistance	EFV	Resistance	
	ETR	Possible resistance	ETR	Possible resistance	9
	NVP	Resistance	NVP	Resistance	
			RPV[c]	Resistance	
4363		No evidence of resistance	ATV[c]	Resistance	
			ATV/R[b]	Possible resistance	25
			NFV[c]	Resistance	
2119		No evidence of resistance		No evidence of resistance	25
11657		No evidence of resistance		No evidence of resistance	10

[a] d4T, stavudine; TDF, tenofovir; AZT, zidovudine; ABC, abacavir; ddI, didanosine; ATV, atazanavir; R, ritonavir; DRV, darunavir; FPV, fosamprenavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; SQV, saquinavir; 3TC, lamivudine; FTC, emtricitabine; EFV, efavirenz; ETR, etravirine; RPV, rilpivirine.
[b] Protease inhibitors administered with low-dose ritonavir for pharmacologic boosting.
[c] Plasma from ViraStim tubes showed additional mutations including mutations that conferred antiretroviral resistance in 3 out of 7 individuals.

patients with starting viral loads of >20 and <1,000 cp/ml, and 8 out of 13 patients with starting viral loads of >1,000 compared to viral loads 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 intrarun variability of viral load assays. Although 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 of <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 receiving suppressive antiretroviral therapy (19). The percentages in the other two nonsuppressed groups were 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 in 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 those in 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 that in the sample tube, we found differences in resistance mutations and amino acid changes compared to those for 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 of antiretroviral drugs in patients treated for more than 2 years with suboptimal drug regimens resulted in the 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 might 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 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 might 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 might be tailored based on sequencing data on the induced PBMC reservoir as described in the present study.

In conclusion, the 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.

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