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Increased mutations in env and pol suggest greater HIV-1 replication in sputum-compared to blood-derived viruses

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Abstract

Objective—Low-level HIV-1 replication may occur during antiretroviral therapy (ART) that suppresses plasma HIV-1 RNA to <50c/mL (suppressive ART). Antiretroviral drugs appear less effective in macrophages and monocytes compared to lymphocytes, both in vitro, and as implied in vivo by greater viral evolution observed during suppressive ART. Our objective was to examine sputum, which is rich in macrophages for evidence of increased HIV-1 replication compared to that in the blood during suppressive ART.

Design—Cross sectional study during suppressive ART. Comparison of HIV-1 DNA sequences derived from induced sputa and peripheral blood mononuclear cells (PBMC).

Methods—Multiple sequences encoding HIV-1 reverse transcriptase, protease, and envelope were generated using single-genome-sequencing. Reverse transcriptase and protease sequences were analyzed for genotypic drug resistance. The evolutionary distances of env sequences from the inferred most recent common ancestor of infection were calculated and CXCR4 co-receptor usage was predicted.

Results—970 bidirectional sequences from 11 individuals were analyzed. HIV-1 env and pol derived from sputa had greater frequency of drug resistance mutations ($P = 0.05$), evolutionary divergence ($P = 0.004$) and tendency for CXCR4 usage ($P = 0.1$) compared to viruses derived from PBMC.

Conclusion—The greater frequency of HIV-1 drug resistance mutations and divergence of HIV-1 env in sputa- compared to PBMC-derived viruses suggests greater HIV-1 replication in the respiratory tract compared to the blood. Characterization of viral evolution over time and by cell-type could identify cells that provide a sanctuary for low-level viral replication in the respiratory tract during suppressive ART.

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Keywords

drug resistance; highly active antiretroviral therapy; HIV-1; lung; macrophages; replication; sputum; virus

Introduction

Twenty [1] to forty-five percentage [2] of individuals starting antiretroviral treatment (ART) have been reported to fail to achieve sustained suppression of viral replication. Even during seemingly effective ART, low-level viral replication has been evident in the blood of a subset of individuals[3-6].While many individuals demonstrate little or no HIV-1 replication in the peripheral blood mononuclear cells (PBMC) during ART [7,8], the slow rate of viral decay during ART suggests replenishment of persisting viral reservoirs [9,10]. Several observations suggest that blood may not be the optimal tissue in which to assess low-level replication. First, plasma HIV-1 sequences during and after ART indicate that PBMC are not the principal source of circulating HIV-1[7,11-13]. Second, mucosal associated lymphoid tissue is a primary target of HIV-1 [14], and HIV-1 induces dramatic changes in the mucosal associated lymphoid tissue [15-17] that are not reflected in the peripheral blood. Lastly, two studies of gastrointestinal tissue suggest that low-level replication can occur during ART that suppresses plasma HIV-1 RNA below the limit of detection [17,18].

HIV-1 replication within specific cells may differ during suppressive ART due to the heterogeneity of intracellular antiretroviral (ARV) pharmacokinetics [19]. In vitro studies of macrophages show poor phosphorylation of nucleoside reverse transcriptase inhibitors to the active form [20], and require higher concentrations of protease inhibitors to inhibit viral replication [21]. In addition, increased viral evolution was observed in monocytes compared to lymphocytes separated from cells collected by leukapheresis during suppressive ART [22]. These observations suggest that tissue macrophages may serve as a relative sanctuary during ART without being readily detected in the peripheral blood.

Consistent with this hypothesis, several studies conducted in the pre-ART era[23-26] observed that HIV-1 DNA from alveolar macrophages was compartmentalized and more divergent from the most recent common ancestor (MRCA) of infection when compared to PBMC-derived virus [23,24]; suggesting that alveolar macrophages and/or lymphocytes in the respiratory tract may be a site of increased viral replication. Most of these specimens were obtained during active pulmonary disease, when increased immune activation and viral replication would be expected. High concentrations of HIV-1 in the lung compared to other organs collected during autopsy [25], and different drug-resistance mutations (assessed by consensus sequencing) in bronchoalveolar lavage compared to blood specimens collected during failing ART [26], also suggest that the lung may be a site of increased or differential HIV-1 replication.

Identifying sites of low-level replication during apparently effective ART may help develop strategies to improve the success of long-term ART [27]. This pilot study examined induced sputa, rich in alveolar macrophages, to gauge the relative amount of HIV-1 replication compared to blood. Sputa and blood were collected from individuals without respiratory symptoms, and viral replication evaluated by analyses of single viral genome-derived sequences of HIV-1 pol, encoding reverse transcriptase (RT) and protease (PR), and envelope (env), to predict drug resistance and gauge viral divergence.

Methods

Participants and specimens

A cross-sectional study of HIV-1 DNA from blood and sputa was conducted at Seattle Children's Hospital, among children >6 years old, with no respiratory symptoms, and receiving ART with plasma HIV-1 RNA <50 copies/mL. Informed consent/assent was obtained in accordance with the Institutional Review Board. Alveolar sputa induced by ultrasonic nebulization (Ultra-Neb99, DeVilbiss, Somerset, PA) of hypertonic (3%) saline was expectorated after four minutes of inhalation, and repeated five times [28]. Sputum was treated with 10% dithiothreitol (Calbiochem, San Diego, CA) for 1 2 h, resuspended in PBS, and quantified (Z1 Coulter Counter, Beckman, Brea, CA). Differential cell counts were performed after Wright-Giemsa staining. PBMC (Accuspin tubes, Sigma-Aldrich, St. Louis, MO) and sputa cell DNA was extracted using Gentra DNA Purification System (Minneapolis, MN).

HIV-1 DNA amplification and quantification

Limiting dilution PCR was performed as previously described [6,29]. Multiplexed first round PCR of pol and env used three sets of primers (RTA/RT1, PRA/PR2, and ED31/BH2), followed by separate second round reactions with RTB/RT4, PRB/PR4, and ES7/ES8 to amplify the RT and PR regions of pol, and the C2-V5 region of env. Alternative primers were occasionally used to optimize amplification, including for the first round: RT2, RTC, Polfo (5′-TCAGAGCAGACCAGAGCCAAC-3′; HXB2 coordinates: 1347 1367), PR5, ED5, ED12, ED3 (5′-TTAGGCATCTCCTATGGCAGGAAGAAGCGG-3′; 5957 5986) and ED14 (5′-TCTTGCCTGGAGCTGTTTGATGCCCCAGAC-3′; 7961 7932); and for the second round: PRC, UHGR, RT3, and Polfi (5′-CAACAGCCCCACCAGAAGAGA-3′; 2153 2173). Cycling conditions and other primers have been previously published [6].

Sequencing

Bidirectional sequencing of PCR products was performed as previously described [6] and submitted to GenBank, accession numbers: FJ446721-FJ447017 and FJ447018-FJ447337.

Sequence analysis

Sequences were assembled, edited, aligned, and screened for hypermutation as described [6]. Sequences with ambiguous bases, indicating the possibility of multiple templates, were discarded. Maximum likelihood phylogenetic trees were constructed using the DiverAnalysis web tool (http://indra.mullins.microbiol.washington.edu/cgi-bin/DIVER/diver.cgi). For each individual, reference sequences from the same subtype were used as an out-group. The inferred sequence at the basal node of each participant's tree was used as an estimate of the MRCA of his/her infection, from which divergence of that participant's extant viral sequences was calculated. Major drug resistance mutations in RT were defined as per the IAS-USA (http://www.iasusa.org). HIV-1 co-receptor usage was predicted using a X4R5 position-specific scoring matrix (PSSM) [30]. Nonsubtype B sequences were also predicted based on the charge at amino acid positions 11 and 25 [30].

Statistical analysis

The average viral divergence and frequency of sequences with drug resistance mutations were calculated for each PBMC and sputum specimen. Because sequences from individuals on suppressive therapy have been shown to regress toward the MRCA [6,31,32], we analyzed average divergence on the assumption that it will be more sensitive to the minority of sequences which had undergone replication. Differences were compared with a Wilcoxon Two-sided Ranked-Sign Test, using the mean PBMC and sputum values from each individual participant as a paired set.

Results

Fourteen children, 7 to 17 years of age, who had been prescribed ART for a median of 6.1 years, were enrolled. Three participants were excluded from analyses; two had sputa with <2 million cells/specimen; and in the third PCR failed to amplify HIV-1. The remaining 11 participants included in the analysis were 55% (6/11) female, 64% (7/11) African American, 18% (2/11) Hispanic, and 9% (1/11) Caucasian. Clinical characteristics of these participants and their sputum specimens are shown in Table 1. Once ART suppressed the plasma HIV-1 RNA to <50 c/mL, low-level viremias were detected on a median of 3% (range 0 – 17) of determinations, at a median of 97 c/mL (range 51 308), and none of 283 HIV-1 RNA determinations was greater than 400 c/mL. Sputa specimens had a median of 16 (range 2 52) × 106 cells, with a median macrophage to lymphocyte ratio of 7.7 (range 3.2 – >100), which did not correlate with concomitant peripheral blood monocyte to lymphocyte ratio (Spearman's rank correlation statistic: $\rho = 0.17$, $P = 0.622$). Eighteen to 58 (median 28) single-genome sequences/gene were generated from each participant's specimens with a total of 970 bidirectional sequences analyzed.

The mean divergence of the sputum-derived HIV-1 env sequences from the MRCA in all 11 participants was greater than PBMC-derived sequences ($P = 0.004$) (Fig. 1a). The mean divergence of all sputa-derived sequences was 9.9% versus 8.0% for all PBMC-derived sequences. This corresponds to a mean of >11 additional nucleotide changes in sputum- compared to PBMC-derived env sequences over the approximately 625 base pair region.

Major protease inhibitor-associated mutations were only found in one participant (J1), whereas mutations associated with resistance to nucleoside analog reverse transcriptase inhibitors (NRTI) were detected in 7 of 11 and two of these also had resistance to nonnucleoside reverse transcriptase inhibitors (NNRTI) (Fig. 1b). All seven of these participants had treatment with mono- or dual-NRTI prior to highly active ART. In 6 of these 7 participants the percentage of sequences with drug resistance mutations was greater in sputum- compared to PBMC-derived sequences and in one (C1) it was equal ($P = 0.05$). Averaging all participants, the mean percentage of sequences with drug resistant HIV-1 was 52% from sputa versus 36% from PBMC.

Env codons associated with the use of the CXCR4 co-receptor (X4 sequences) were found in 6 of 11 participants. In five of the six, the percentage of X4 sequences was greater in sputa- compared to PBMC-derived sequences ($P = 0.1$), with overall means of (41%) versus (31%). The PSSM algorithm is less well validated in nonsubtype B virus. Scoring of basic amino acids at positions 11 and 25 for the two participants with nonsubtype B virus did not change the predicted phenotype of any sequences from I2 (subtype F) but decreased the percentage of predicted X4 phenotypes proportionally in both sputum- and PBMC-derived sequences from F1 (subtype D).

Discussion

During suppressive ART, features of HIV-1 sequences indicative of increased viral replication were more common in viruses derived from sputum compared to PBMC. Specifically, sputum-derived HIV-1 env had a greater mean divergence from the ancestor of infection, an increase in the frequency of drug resistance mutations in HIV-1 RT, and a trend to greater X4 genotype. These changes are characteristic of ongoing viral replication in untreated persons [33,34]. Detection of evolution in two genes (pol and env) under different selective forces (antiretrovirals and host immunity) strengthens our evidence for increased viral replication in the sputa-derived viral sequences.

A different rate of viral replication in the respiratory tract cells relative to the blood is a straightforward explanation for the observed differences. However, unequal selective pressure and rates of HIV-1 infected cell turnover may also have contributed to the disparity. In this cross-sectional study, we cannot determine when or where these differences originated. Longitudinal studies should provide insight into whether viral replication and/or selection are ongoing during suppressive ART.

Inflammatory cytokines are associated with increased HIV-1 replication [35], and may have contributed to the differences between viruses we amplified from the sputum and blood, as well as those with pulmonary infections in previous studies [23,24,26]. We reason that increased immune activation in cells within the respiratory tract mucosa and associated lymphoid tissue, even in the absence of respiratory symptoms, could enhance viral replication and/or the proliferation of infected cells compared to the blood.

Given that macrophages were more prevalent than lymphocytes in all sputa, and that the HIV-1-DNA loads we measured in sputa were similar to published levels in purified alveolar macrophages [24,36], it is tempting to speculate that the observed differences are due to HIV-1 from alveolar macrophages. However, recently published data suggests that the bulk of HIV-1 DNA in bronchoalveolar lavage specimens comes from lymphocytes [37,38]. This suggests that the differences in HIV-1 sequences we derived from sputum versus blood may be due to sampling a different population of cells associated with the respiratory tract mucosa.

In summary, increased viral divergence and drug resistance in sequences derived from sputa compared to the blood indicates relatively greater HIV-1 replication in the respiratory tract. Longitudinal characterization of viral evolution by sputa cell-type should determine whether specific respiratory tract cells provide a sanctuary from ART and allow low-level viral replication during ART that appears suppressive by blood tests.

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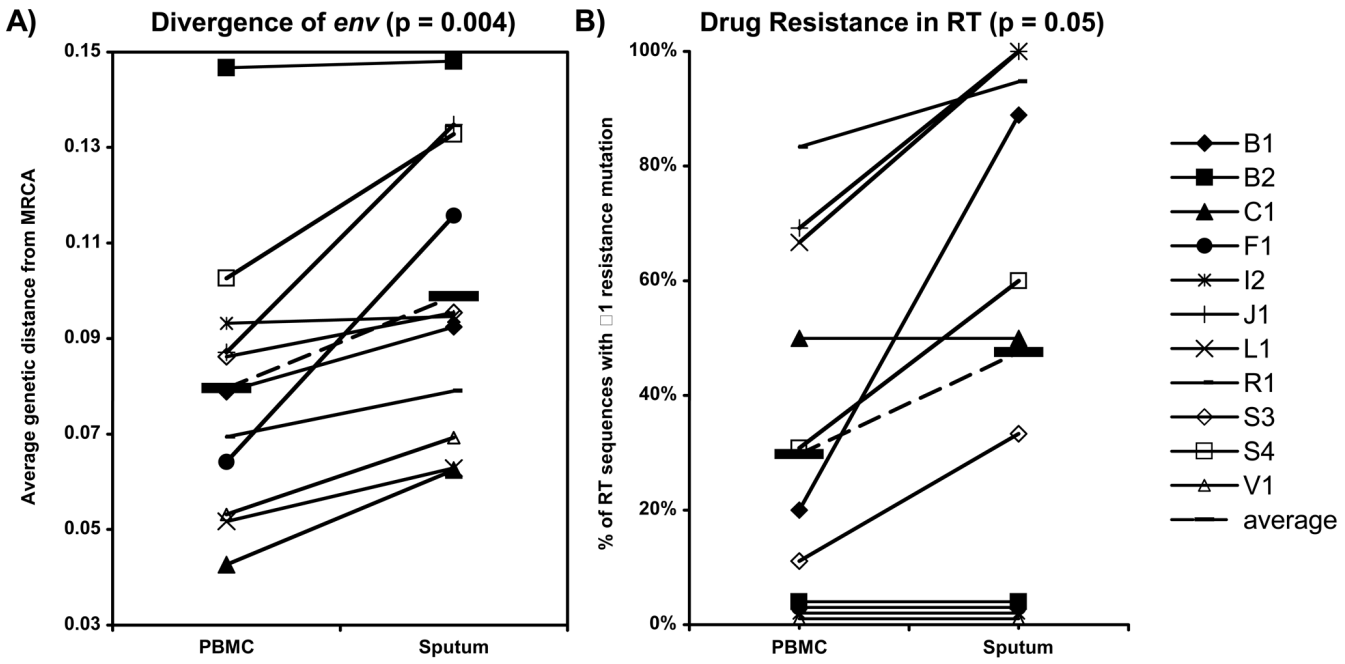

Fig. 1.

HIV-1 sequences derived from induced sputa compared to those from peripheral blood mononuclear cells (PBMC).

The mean value for each participant's sequences is represented by a symbol (see key to right). Plots show (a) HIV-1 mean env divergence from the ancestor of infection, and (b) frequency of RT sequences with ≥1 drug resistance mutation. Values from each participant's sputum and PBMC are connected with a line. The mean of all participants' values is shown for each parameter (bold hashes connected by dashed line). P values refer to the differences between sputum and PBMC values using Wilcoxon Two-sided Ranked-Sign Test to compare the paired mean values from each individual. Values corresponding to 0% on panel B are splayed slightly for better visualization.

Table 1

Characteristics of participants, sputum, and the number of sequences analyzed.

	[0, 2–6]Clinical Characteristics and Blood Values				[0, 7–9]Sputum Analyses*			
			[0, 4–5]Plasma HIV-1 RNA determinations during ART		HIV-1 DNA copies per 106 CD4+ in PBMC	# of Sputum Cells (× 106) Collected	Macrophage to Lymphocyte Ratio in Sputum, (Bloodc)	HIV-1 DNA copies per 106 Macrophages and Lymphocytes
Participant	**ART Regimena**	**Years on ART**	**# >50/>400 copies/mL**	**Total #**				
B1	A	7.1	0/0	40	194	52	3.5 (0.17)	156
B2	A	6.2	3/0	31	593	21	3.2 (0.26)	165
C1	A	7.0	0/0	22	126	11	5.2 (0.16)	342
F1	A	3.8	5/0	30	1163	4	7.7 (0.34)	27
I2	B	1.5	0/0	8	614	38	5.0 (0.19)	18
J1	B	4.2	2/0	23	451	19	21.3 (0.20)	13.3
L1	A	7.1	2/0	37	262	5	4.7 (0.29)	230
R1	B	6.6	1/0	25	228	16	>100:1 (0.42)	90
S3	A	1.6	0/0	15	116	3	>100:1 (0.21)	20
S4	A	0.8	3/0	21	764	2	20.0 (0.23)	5
V1	A	5.8	1/0	31	2314	38	15.4 (0.16)	79
Median		5.8	1/0	25	451	16	7.7 (0.21)	79
Total			17/0	283				

ART, antiretroviral therapy; PBMC, peripheral blood mononuclear cells.

*Included a median of 34.5% (range 5 – 70.5%) squamous epithelial cells and 29% (range 3 – 82%) neutrophils.

[a]Antiretoviral Regimens: A = three class combination with nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitor, and protease inhibitor; B = two class combination with nucleoside reverse transcriptase inhibitors and protease inhibitors (lopinavir with ritonavir).

[b]estimated by QUALITY program [29] applied to limiting dilution PCR of env.

[c]ratio of monocytes to lymphocyes in the concomitant peripheral blood.