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Recovery of Replication-Competent Residual HIV-1 from Plasma of a Patient Receiving Prolonged, Suppressive Highly Active Antiretroviral Therapy▽

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The clinical significance of persistent residual viremia in patients on prolonged highly active antiretroviral therapy (HAART) is not clear. Moreover, it remains to be demonstrated whether residual viremia consists of viruses capable of spreading infection *in vivo* upon termination of therapy. Using residual viral RNAs (vRNAs) isolated from a HAART-treated patient's plasma, we cloned full-length viral genomes and found that most of them could produce infectious, replication-competent HIVs when transfected into TZM-bl cells, suggesting that residual viruses produced in the absence of therapy can initiate fresh cycles of infection and spread in host cells. The data further indicate that residual viremia may pose a major concern with regard to the emergence of drug-resistant HIVs during periods of low adherence to therapy.

The combination of antiretroviral drugs used in current highly active antiretroviral therapy (HAART) regimens has been highly effective in suppressing viral load to below the detection limit (~50 to 75 copies of HIV RNA per ml of plasma) of clinical viral load assays (9, 10, 16). However, more-sensitive laboratory tests have frequently detected fewer than 50 copies of viral RNAs (vRNAs) in the plasma of HAART-treated patients (3, 7, 11, 12, 17, 24–26). These extremely low viral loads (termed residual viremia) persist over many years, even though patients continue to receive long-term suppressive therapy (17, 24). In addition, intensification of HAART, using an HIV integrase inhibitor, cannot reduce the levels of this persistent viremia (6, 19). The clinical significance, as well as the cellular source, of residual viremia during HAART remains unclear.

Recently, we and others showed that residual viruses are likely produced in an unknown cell type(s) different from CD4 T cells circulating in patients' blood (4, 31). Most of these residual viruses do not possess drug-resistant mutations in their *pol* genes (12), and it is presumed that they remain immature in the presence of the protease inhibitor used in the HAART regimen. Accordingly, in the absence of therapy, the newly produced residual viruses should mature and start new cycles of infection and spread in patients' bodies. However, the possibility of such a scenario has not been demonstrated directly by showing that replication-competent viral genetic materials are represented in the pool of residual vRNAs in plasma during suppressive therapy. Although a number of investigators in the past successfully isolated replication-competent HIVs from peripheral blood mononuclear cells (PBMCs) of patients treated with suppressive HAART (5, 8, 37), the isolation of replication-competent viruses directly from such patients' plasma has not been reported.

To this end, we generated molecular clones of residual viruses, starting with plasma vRNAs isolated from a patient who was chronically infected with HIV and treated with antiretroviral therapy for many years. This patient, a 59-year-old male, has been infected with HIV since the mid-1980s and started receiving antiretroviral therapy in 1989. He was prescribed the combination of lopinavir-ritonavir, tenofovir, and efavirenz in February 2002 and has continued on this regimen, but he had previously taken other antiretrovirals with suboptimal adherence resulting in treatment failure (further information was not available). His viral loads were measured every 3 to 4 months between July 2005 and August 2007 (study analysis period), using a commercial HIV RNA load assay (Bayer Corporation; the detection limit was 75 copies per ml). He had a mean CD4 count of 367 and stable viral loads of <75 copies per ml during these 2 years (except for one blip of 118 copies per ml in November 2006, which might be due to the assay variation reported by others [21]). Prior to blood sample collection in August 2007, he was asked to sign a consent form approved by the University of Texas Medical Branch (UTMB) institutional review board for participation in this study.

Plasma was isolated from his blood and subjected to ultracentrifugation methods described previously for pelleting residual viruses (15, 31). Residual vRNAs were isolated from the virus pellet, using a QIAamp viral RNA mini kit (Qiagen). To ensure removal of any contaminating DNAs from virus-infected blood cells, we carried out RNase-free DNase I treatment on the column at room temperature for 30 min according to the manufacturer's instructions.

Fifteen microliters (the plasma equivalent of 8 ml) of eluted vRNAs were taken as templates for reverse transcription (RT)-PCR amplification of various segments of the HIV genome (shown schematically in Fig. 1A). Amplification was performed by using a Superscript III One-Step RT-PCR kit (Invitrogen) for long templates. All amplifications were carried

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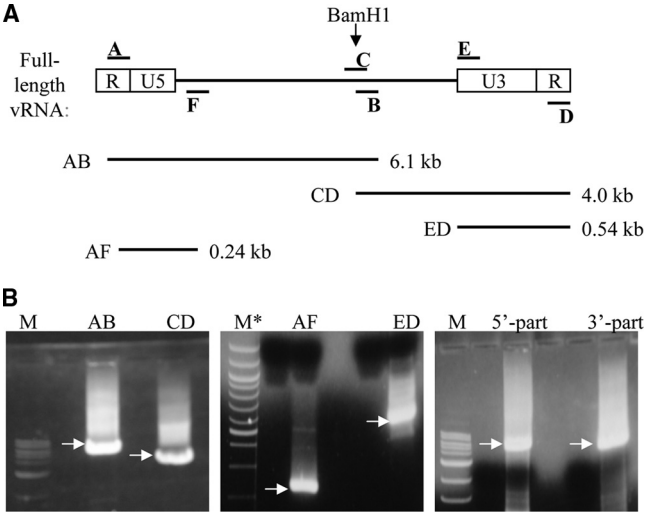

FIG. 1. Cloning of the residual-virus genome. (A) Schematic diagram of HIV genome segments amplified by nested RT-PCR, using residual vRNA as the template. U3, R, and U5 are regions of the two long terminal repeats of HIV. The small bars designated A, B, C, D, E, and F represent the approximate primer binding sites. AB, CD, ED, and AF represent the amplified fragments of the specified sizes. (B) All amplified fragments (indicated by white arrows) were electrophoresed in 1% agarose gel. Lane markers correspond to the amplified segments shown in panel A. Lanes M and M* are 1-kb and 100-bp markers (New England Biolabs), respectively.

out by using thermostable DNA polymerases with proofreading activity and appropriate primers and reaction conditions (not shown). The product was diluted 10-fold, and a nested amplification step was performed by using an Expand high-fidelity PCR kit (Roche). The amplified fragments are shown in Fig. 1B (see left two gels). We also performed nested PCR without the reverse transcription step but could not amplify the fragments (data not shown), which demonstrated that the amplified fragments were not due to any contaminating DNA in the vRNA preparations.

The amplified DNA fragments were eluted from agarose gels and treated with T4 DNA polymerase to remove any 3′-A overhangs incorporated during PCR, making the fragments blunt ended. The smaller fragments, AF and ED, were joined with the larger fragments, CD and AB, respectively (Fig. 1A), in an overlapping PCR method. The resulting amplified fragments (the 5′ part and the 3′ part) shown in the gel on the extreme right of Fig. 1B were cloned into a pCR-XL-TOPO(TA) vector (Invitrogen). We checked the junctions of various fragments by sequencing to make sure that there was no defect incorporated during the process of joining them by overlapping PCR. From the restriction digestion analyses, we found that residual viral DNAs (vDNAs) possessed a unique restriction site for BamHI located in the overlapping region of the 5′ and 3′ parts of the amplified viral genome (Fig. 1A). Taking advantage of this unique site, we joined one 5′ part with five different 3′ parts in the correct orientation to obtain five clones of the full-length viral genomes as plasmid forms.

To check whether these recombinant viral clones (named C1P, I-1, I-2, N-1, and N-2) are capable of producing infectious virus, ~2 μg each of the five vDNA clones were transfected separately into TZM-bl cells (36) in 6-well plates by using FuGene HD reagent (Roche) and incubated overnight in a CO_2 incubator at 37°C. On the following day, the media were replaced with 2 ml of fresh media and incubated for another 48 h. Then the supernatants were tested for p24 production by using an HIV p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Advanced BioScience Laboratories, Inc.). All vDNA clones produced high levels of HIV p24 after transfection, but clone N-2 produced ~40-fold-lower levels of p24 in the culture supernatant than the other clones (data not shown). In order to check if p24-positive supernatants possess infectious viral particles, 1 ml of the culture supernatants was added to freshly prepared TZM-bl cells (an indicator cell line expressing CD4, CCR5, and CXCR4 receptors for HIV) in 24-well plates and incubated for 48 to 72 h. Then cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in a multinuclear activation of a galactosidase indicator (MAGI) assay (36). The uninfected TZM-bl cells were considered background controls. We found that the supernatants derived from clones C1P, I-1, and I-2 produced many (>500) blue cells, whereas the other two clones (N-1 and N-2) produced none. Therefore, 60% (3/5) of our vDNA clones could produce infectious virus particles upon transfection into human cells, and the data demonstrated for the first time that at least a portion of residual viruses in this patient carry genetic materials capable of producing infectious virus particles.

Analyses of viral sequences revealed that clones C1P and I-1 apparently had no gross defects in any of the viral genes, whereas clone I-2 had a nonsense (stop codon) mutation at codon 33 of the *nef* gene. Of the other two clones, clone N-2 had a loss-of-function missense mutation (C22R) in the *tat* (exon 1) region (30) and a nonsense mutation at codon 507 in the *env* (gp120) gene, likely causing the noninfectivity observed in MAGI assays. The other noninfectious clone (N-1) did not have any major defect in the viral open reading frames (ORFs); however, when we compared sequences of the 3′ parts of all five clones, we found that one of the mutations, D368G, which is located in the C3 region of Env and known to be important for CD4 binding (22, 23), might be responsible for its noninfectivity in TZM-bl cells.

To test whether the cloned residual viruses can replicate in activated PBMCs, freshly isolated PBMCs from a normal donor's blood were stimulated with 4 μg/ml of phytohemagglutinin (PHA) for 2 days. Then fresh supernatants (~10 to 100 ng of p24 per ml) harvested from each vDNA-transfected TZM-bl cell culture were added to the stimulated PBMCs (2×10^6) in 24-well plates and incubated overnight at 37°C in a CO_2 incubator. Cells were washed three times the next day and cultured in fresh media with interleukin-2 ([IL-2] 40 U/ml) for 2 weeks. Two laboratory strains (MCK and JRCSF) were prepared similarly by transfecting TZM-bl cells with their DNA clones and were used equivalently as positive controls for infection. Uninfected (mock-stimulated) PBMCs were kept as negative controls. Supernatants from PBMC cultures were collected on the days indicated in Fig. 2 and tested for HIV p24 levels by using a p24 ELISA kit (Advanced BioScience Laboratories). The increases in p24 levels in the culture supernatants as the days progressed (Fig. 2) demonstrate that residual viruses produced from C1P, I-1, and I-2 clones can infect and replicate in activated PBMCs, similar to findings for TZM-bl cell infections, whereas the other two clones (N-1 and N-2) could not. Later

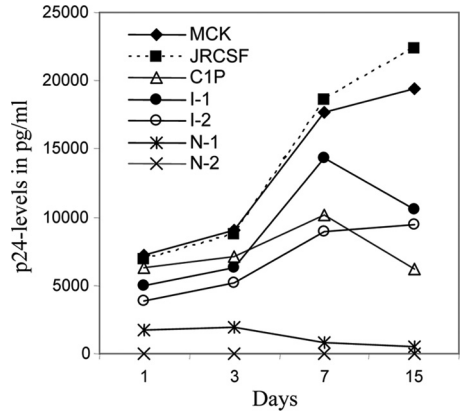

FIG. 2. Replication of residual viruses in activated PBMCs. C1P, I-1, and I-2 are infectious viral clones, but N-1 and N-1 are noninfectious clones. MCK and JRCSF are standard laboratory strains.

we found that C1P, I-1, and I-2 viruses could also infect and replicate in purified, activated primary CD4 T cells *in vitro* (data not shown).

To determine the coreceptor usage of cloned residual viruses, we analyzed the V3 region by using Web-based programs (2) that predicted that these viruses would use the CCR5 coreceptor to infect a target cell. To verify this prediction, we harvested fresh supernatants from TZM-bl cells transfected with three infectious vDNA clones (C1P, I-1, and I-2) and added these to Ghost ($CD4^+CCR5^+$ or $CD4^+CXCR4^+$) indicator cell lines (NIH AIDS Reagent Program) (20). In infected cells, viral Tat proteins expressed intracellularly induce expression of the green fluorescent protein (GFP) gene under the control of the HIV-2 long terminal repeat (LTR). By analyzing GFP expression under a fluorescence microscope, we found that all three residual viruses could infect Ghost $CD4^+CCR5^+$ cells but not Ghost $CD4^+CXCR4^+$ cells (Fig. 3A). This finding is reflected by the accumulation of HIV p24 in the culture supernatants (Fig. 3B). As expected, CCR5-blocking antibody (clone 2D7; BD Biosciences) blocked infection by these viruses of $CCR5^+$ Ghost cells (data not shown). Therefore, these data demonstrate that these cloned infectious residual viruses are CCR5 tropic, as has been suggested previously by others (1). However, it was recently reported that a proportion of residual viruses in the plasma of some patients on HAART could also use the CXCR4 coreceptor (18).

We observed previously with several patients on suppressive therapy that residual plasma viruses and CD4 T-cell-associated viruses were compartmentalized separately (31), which was also found recently by others (4). This finding suggests that residual viruses might be produced in an as-yet-unknown cellular source different from circulating CD4 T cells in patients receiving suppressive HAART.

We also analyzed viral *tat* sequences isolated from CD4 T cells and plasma of the patient referred to in this study by using phylogenetic methods. The nucleotide sequences were aligned by using CLUSTALW algorithms, and the phylogenetic trees were constructed by using the neighbor-joining method (32) implemented in the MEGA4 package (34). The evolutionary distances were computed by using the Tamura-Nei method (35). Although a few sequences isolated from CD4 T cells were found to commingle with the plasma-derived sequences in the phylogenetic trees, the majority of the sequences isolated from both sources appeared to cluster separately (Fig. 4). The compartmental analyses performed by using the Slatkin-Maddison test (33) implemented in the HyPhy package (29) showed that all these sequences were compartmentalized in a source-specific manner ($P < 0.001$), similar to the results of our previous study (31).

Analysis of the viral *pol* gene represented in our 5 clones by using the HIVdb program (Stanford University) showed that it possessed the mutations M46I and L90M, which might confer low-level resistance to lopinavir-ritonavir (13). Also, the other mutations, such as M41L and T215Y, may result in an intermediate level of resistance to tenofovir (14, 27, 28). However, no resistance mutations were found for efavirenz, which was also used in the HAART regimen, indicating that residual viruses were mostly sensitive to the combination of antiretroviral drugs used by this patient.

The data presented here demonstrate that at least a portion of the residual plasma viruses in this patient remain capable of

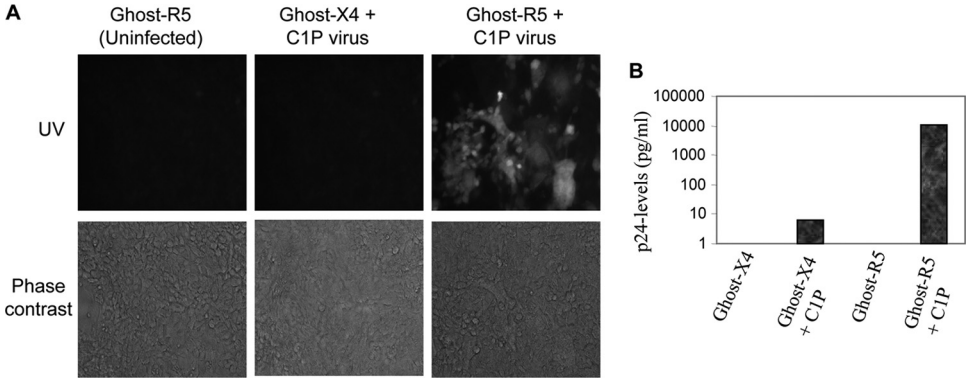

FIG. 3. Coreceptor usage of residual viruses. Residual virus-infected indicator Ghost cells were visualized under a UV microscope. (A) GFP-expressing green cells were observed only when Ghost cells expressed CCR5 as an HIV coreceptor (panels on right). (B) Increase in the levels of HIV p24, measured by an HIV-1 p24 antigen capture assay (Advanced BioScience Laboratories, Inc.) in culture supernatants of infected Ghost cells 72 h after infection. Only data for C1P, one of three infectious residual-virus clones, is shown here. Standard laboratory strains JRCSF (R5 tropic) and MCK (X4 tropic) were used as positive controls for infection (data not shown).

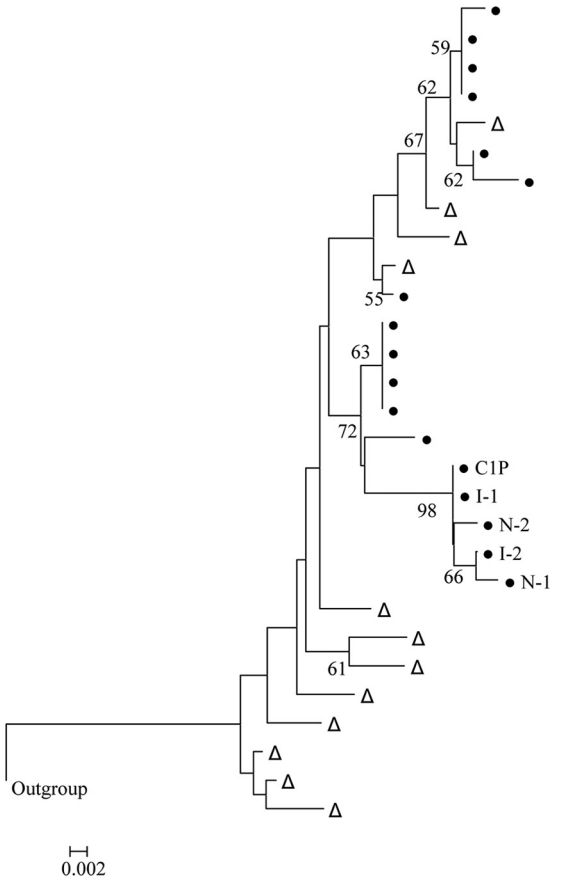

FIG. 4. Evolutionary relationships among the *tat* sequences isolated from plasma (●) and CD4 T cells (△). The reliability of the internal branches was estimated from 1,000 bootstrap replicates, and the values were expressed as percentages, which are shown next to the branches. Only bootstrap values higher than 50% are shown. Solid circles (●) adjacent to C1P, I-1, I-2, N-1, and N-2 represent *tat* sequences from the corresponding molecular clones of residual viruses.

replicating in the absence of HAART. If this is also true for other patients, then the persistent residual viremia should contribute significantly to the evolution of drug resistance during HAART, particularly during low adherence to therapy. Therefore, identification of the cellular source(s) for residual viremia and its underlying mechanisms of persistent production should remain important goals for designing new therapeutic approaches to completely suppress virus production and to target the cellular source(s) for elimination.

Nucleotide sequence accession numbers. All five vDNA clones were sequenced and deposited in GenBank under accession numbers GU733713 to GU733717.

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