Increased Replication of HIV-1 Minor Variants during Hematopoietic Stem-Cell Mobilization with Filgrastim

Thomas B. Campbell, Eric Rapaport, Robert T. Schooley, and Daniel R. Kuritzkes

The hypothesis that filgrastim (r-met-huG-CSF) activates replication of minor variants of human immunodeficiency virus type 1 (HIV-1) was tested by analysis of plasma quasi-species composition in 7 subjects in whom plasma HIV-1 RNA had increased during filgrastim treatment. Inferred phylogenetic trees of env sequences from 3 subjects during filgrastim treatment contained unique intrasubject subclusters that shared a most recent common ancestor with the baseline HIV-1 quasi species. Genotypes in the unique subclusters were not detected before filgrastim treatment, yet they composed 40%–70% of the plasma quasi species during treatment. The minority variants that appeared in 1 subject were more distantly related to plasma quasi species present 5 years before filgrastim treatment than were the majority of the pretreatment plasma quasi species. These findings provide evidence that increased HIV-1 replication during filgrastim treatment was associated with activation of HIV-1 variants that, before filgrastim treatment, were minor components of the plasma quasi species.

The diverse genotypes of HIV-1 that are typically present in the plasma of an infected individual compose a complex population referred to as a “quasi species” [1, 2]. The HIV-1 quasi species present in plasma closely mirror the actively replicating HIV-1 genotypes, because of rapid equilibration between virus-producing tissue compartments and the plasma [3, 4]. The plasma quasi species are derived predominantly from productively infected cells in lymphoid tissue [5], but other body compartments, such as brain, semen, cerebrospinal fluid, and lung tissue, harbor distinct HIV-1 genotypes [6, 7].

Factors that alter the level of immune activation in various tissue compartments harboring HIV-1 can shift the composition of the plasma quasi species. Abrupt changes in quasi-species composition accompany the increase in HIV-1 replication that occurs in response to immunization with recall antigens, possibly because of induction of virus production in a subset of latently infected cells [8]. In contrast, generalized immune activation with interleukin-2 (IL-2) transiently increases HIV-1 replication, but the virus induced by IL-2 most closely resembles the pretreatment plasma quasi species [9].

Adult AIDS Clinical Trials Group (AACTG) Study 285 evaluated the effect that hematopoietic stem cell (HSC) mobilization with filgrastim (r-met-huG-CSF) treatment has on HIV-1 replication in infected persons [10]. In 9 of 18 subjects, plasma HIV-1 RNA levels increased 4- to 100-fold above baseline between days 4 and 7 and returned to baseline by day 27 in 9 of 18 subjects [11]. Significant increases of plasma HIV-1
RNA levels occurred in 5 subjects who received 3-drug antiretroviral therapy during filgrastim treatment. The treatment regimens of these patients and of all of the other patients in the present study have been reported elsewhere [11]. The rapid increase in plasma HIV-1 RNA levels during HSC mobilization suggested that viral replication was stimulated in a large pool of cells. However, whether this increase in HIV-1 replication represents a generalized increase in viral production by cells and tissues contributing to the dominant quasi species before filgrastim administration or represents activation of a unique reservoir of HIV-1 is not known.

In the present study, we have evaluated the hypothesis that filgrastim activated a unique reservoir of HIV-1 by determining the effects that HSC mobilization has the phylogenetic relatedness of the plasma HIV-1 quasi species. If, like IL-2 therapy, filgrastim causes a nonspecific activation of HIV-1 replication, little change in the composition of the plasma quasi species would be expected. However, if filgrastim stimulates HIV-1 production in a unique population of HIV-1–infected cells (similar to the effect produced by antigen-specific stimulation), distinct changes in the plasma quasi-species composition would be expected.

SUBJECTS, MATERIALS, AND METHODS

Study population. This study followed the US Department of Health and Human Services Guidelines for human experimentation and was approved by the Colorado Multiple Institutional Review Board. Eighteen HIV-1–seropositive AACTG 285 participants received subcutaneous 10–μg/kg/d injections of filgrastim (Neupogen; Amgen) on study days 1–7. On days 4 and 5, peripheral-blood mononuclear cells (PBMCs) were harvested by leukapheresis. For 7 of the 9 subjects who had significant increases in plasma HIV-1 RNA levels during HSC mobilization [10, 11], plasma specimens collected at baseline (study day –3 or 0) and at the time of peak plasma viremia (study day 4, 5, 6, or 7) were analyzed; plasma specimens from the remaining 2 subjects were not available. Additional archived plasma specimens collected from 1 subject (051) during participation in previous AACTG studies, collected 2–5 years before entry into AACTG 285, were also analyzed. Characteristics of these subjects entered into the study have been described elsewhere [10, 11].

Nucleotide-sequence analysis. The C2-V3 region of env was analyzed by reverse-transcription polymerase chain-reaction (RT-PCR) amplification and clonal analysis of PCR products. Viral RNA was extracted from 0.5-mL aliquots of plasma. If the measured amount of plasma RNA was <1000 copies/mL, multiple 0.5-mL aliquots were combined and plasma virus was concentrated by centrifugation before extraction of RNA. For each plasma sample analyzed, extracted RNA was aliquoted in separate quadruplicate RT-PCR with nested oligonucleotide primers designed to amplify a 400-nt segment of the env C2-V3 coding region, as described elsewhere [12]. PCR products were purified by electrophoresis on an ethidium bromide–stained agarose gel. Equal amounts of PCR product from each of the quadruplicate RT-PCRs were pooled, ligated into pCR2.1 TOPO TA cloning vector (Invitrogen), and transformed into Escherichia coli DH5α. The presence of an insert of the correct size was confirmed by digestion with EcoRI, and the nucleotide sequence of the insert was determined by automated DNA sequence analysis (Applied Biosystems). All nucleotide-sequence determinations used both forward and reverse oligonucleotide primers, to obtain the complete sequence of each insert in both the forward and the reverse directions. At least 10 clones were analyzed from each sample.

The nucleotide sequence of HIV-1 reverse transcriptase (RT) and protease (PR) was determined by automated population-based sequence analysis of the plasma quasi species, by the TruGene HIV-1 Sequencing Kit and the OpenGene DNA Sequence Analysis System (Bayer Nucleic Acid Diagnostics) [13]. Clonal analysis of HIV-1 RT was conducted by RT-PCR amplification, as described elsewhere [13], followed by ligation of the PCR product into pCR2TOPO (Invitrogen). Automated nucleotide sequence analysis of HIV-1 RT molecular clones was preformed with both forward and reverse primers; 10 clones from each time point were analyzed.

All RT-PCRs included samples that contained no RNA (negative control) and samples that contained either 10 or 100 copies of pNL4-3 DNA (positive controls). DNA sequences were edited manually by Sequencher 4.0.5 (Gene Codes) and Valigned by CLUSTAL W in Bioedit 5.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic trees of the aligned, edited sequences were constructed to rule out either cross-contamination between samples or contamination by nucleic acids from laboratory HIV-1 isolates [14]. Amino acid sequences were predicted by Bioedit, after stripping of gaps, and were aligned by CLUSTAL W.

Genetic relationship of pre- and posttreatment quasi species. The genetic relationship between plasma HIV-1 before and after stem-cell mobilization was determined by construction of inferred phylogenetic trees of either env C2-V3 or RT nucleotide sequences. Genetic distances were calculated by DNADIST, and trees were constructed by neighbor-joining analysis of data generated by DNADIST. Protein distances were calculated by PRODIST. Graphic depiction of inferred trees was performed by Treeview 1.5 (http://www.taxonomy.zoology.gla.ac.uk/rod/rod.html). Bootstrap analyses were performed by Seqboot software in the Phylip 3.6 package (http://evolution.genetics.washington.edu/phylip.html). Bootstrap values for neighbor-joining trees were determined by comparison of 1000 replicate trees with Seqboot; bootstrap values ≥70% were considered to be significant.
Figure 1. Relationship of HIV-1 env sequences from plasma RNA of 7 subjects immediately before filgrastim treatment (entry) and at the time of peak plasma HIV-1 RNA during filgrastim treatment (+G). Each subject’s identification number is shown in boldface to the right of that subject’s phyletic group. Intrasubject phyletic subgroupings of ≥3 sequences from the time of filgrastim treatment are highlighted. Reference sequences were obtained from GenBank. Bootstrap values shown are the percentage of times that the sequences distal to the node grouped together in 1000 replicate trees. Arrows indicate most recent common ancestors shared by intrasubject phyletic subgroups.
Changes in the plasma HIV-1 quasi-species composition during filgrastim treatment. The C2-V3 coding region of env was successfully amplified from plasma samples obtained at baseline and peak-viremia specimens from all 7 subjects. The env clones from each subject formed unique phylectic clusters that were distantly related to common laboratory strains of HIV-1 and that were distinct from all other subject clusters (figure 1). All of the unique groupings of sequences from individual subjects were supported by bootstrap values ≥89%.

Subcluster groupings of 4 or more env sequences distinct from the sequences present at baseline were present within the individual phylectic clusters of env sequences obtained from 5 subjects (patients 146, 051, 745, 147, and 148; see highlighted sequences in figure 1) at the time of peak viremia. Such subclusters were not detected in samples from subjects 219 and 743 during filgrastim treatment. For each subject, the env sequences that did not group within distinct subclusters were closely related to and intermixed with the baseline plasma quasi species in the inferred phylogenetic tree.

Three of the distinct intrasubject subcluster groupings in the inferred phylogenetic tree (figure 1; subjects 051, 147, and 149) were supported by bootstrap values ≥70% and therefore met all criteria for unique subclusters associated with filgrastim treatment. In these subjects, peak viremia during filgrastim treatment was 21,000–60,000 copies/mL, an increase of 17,000–60,000 copies above baseline (table 1). Because 40%–70% of the env sequences during HSC mobilization grouped into unique subclusters, 8,400–42,000 copies of plasma HIV-1 RNA/mL was attributable to the unique virus that appeared during filgrastim treatment.

On the basis of the inferred phylogenetic tree (figure 1), the unique env subclusters from subjects 051, 147, and 149 were predicted to share a most recent common ancestor (MRCA) with the baseline plasma quasi species. The genetic distances between the MRCA of subjects 147 and 149 and the unique env sequences, and between the MRCA and the entry quasi species, were similar (P = .33 and .78, respectively; Mann-Whitney test). For subject 051, the env sequences associated with filgrastim treatment had greater genetic distances to the MRCA than did the entry plasma quasi species (P = .006; Mann-Whitney test). Although plasma env sequences from subject 146 during filgrastim treatment also shared an MRCA with the pretreatment plasma quasi species (figure 1), the bootstrap values for the inferred tree for these sequences did not meet the criteria for a unique subcluster.

Filgrastim-stimulated replication of minor variants in the plasma quasi species. To assess the genetic relationship between unique HIV-1 sequences that appeared during filgrastim treatment and minor variants present in the plasma quasi species at baseline, additional plasma samples collected from subject 051 at study entry (days −3 or 0) and at 3, 4, and 5 years before study entry were analyzed; archived specimens were not available for the other subjects. We constructed an inferred phylogenetic tree of env sequences from subject 051 that included 30 sequences derived from plasma collected at study entry; 30 sequences derived from plasma collected 3, 4, and 5 years before study entry; and 10 sequences derived from plasma collected during HSC mobilization (figure 2). All sequences from subject 051 formed an MRCA that was distantly related to common laboratory strains of HIV-1 (blackened arrow in figure 2). All sequences from samples collected at entry and during filgrastim treatment were grouped in 1 of 3 unique phylectic subclusters supported by significant bootstrap values (subclusters I–III in figure 2). Six of the env sequences derived from plasma collected on day 5 of filgrastim treatment formed a unique phylectic grouping within subcluster I (highlighted sequences in figure 2) and shared an MRCA with the rest of
Figure 2. Relationship of HIV-1 env sequences from subject 051. Sequences were generated from plasma RNA collected immediately before (entry [i.e., days −3 or 0]), during (+G), or at varying times in the distant past, before filgrastim treatment (−3y, −4y, and −5y). Intrasubject phyletic subgroupings of ≥3 sequences from the time of filgrastim treatment are highlighted. Reference sequences were obtained from GenBank. Bootstrap values shown are the percentage of times that the sequences distal to the node grouped together in 1000 replicate trees. The blackened arrow indicates the most recent common ancestors (MRCA); the unblackened arrow indicates the MRCA of the unique sequences from filgrastim treatment and of a subgroup of the plasma quasi species at entry. Three subgroups (I–III) formed by the entry plasma quasi species and the plasma quasi species 5 years before study entry (−5 y) are indicated. The unique HIV-1 genotypes induced by filgrastim are all contained within subgroup I, which is most closely related to a single genotype present in the quasi species 4 years before study entry (04y).
The genetic relatedness of HIV-1 RT is similar before and after filgrastim treatment. Subject 051 began antiretroviral treatment with zidovudine (ZDV) monotherapy in 1992, 5 years before entry study. This subject subsequently received ZDV plus zalcitabine (ddC); indinavir (IDV) monotherapy; and ZDV plus lamivudine (3TC) plus IDV, which was continued throughout the present study (figure 4A).

To determine whether the HIV-1 induced by filgrastim treatment had evolved in response to prior or current antiretroviral therapy, the RT and PR genes were examined for the presence of amino acid substitutions associated with antiretroviral drug resistance. Because prior antiretroviral-treatment history was available only for subject 051, this analysis was performed only for this subject. Mutations associated with resistance to ZDV (L210W and T215Y), 3TC (V118I and M184V), and IDV (L10I, V32I, A71V and V82A) were detected by direct sequence analysis (figure 4B). The same mutations were detected in the sample obtained from subject 051 at the time of peak viremia during filgrastim treatment (study day 5). The results of population-based sequence analysis were confirmed by sequence analysis of molecular RT clones derived from entry plasma (study day 0) and from plasma collected during filgrastim treatment (study day 5); drug-resistance mutations at RT codons 184, 210, and 215 were present in all 10 molecular clones derived from entry plasma samples and in all 10 molecular clones derived from plasma collected during filgrastim treatment (figure 4C); 9 of 10 entry clones and 7 of 10 filgrastim-treatment clones contained the V118I mutation.

To assess the genetic relatedness of RT sequences present in subject 051 at baseline and during filgrastim treatment, an inferred phylogenetic tree was constructed (figure 5). All RT sequences from subject 051 formed a phylogenetic group that was distantly related to common laboratory strains of HIV-1. All entry and filgrastim-treatment sequences were closely related and intermixed. No unique intra-subject phylogenetic subclusters were detected.
Predicted V3-loop amino acid sequence and coreceptor usage. The predicted amino acid sequence of the HIV-1 V3 loop was determined for each env clone derived from plasma samples collected at baseline and during filgrastim treatment. The unique intrasubject phyletic subclustering in the inferred phylogenetic tree of DNA sequences shown in figure 1 were also observed in a phylogenetic tree constructed on the basis of amino acid sequences (not shown). The env V3 loop of all clones from each subject contained either negatively charged or neutral amino acid residues at positions 11 and 25, which is consistent with CCR-5 coreceptor utilization [15–17]. The predicted net charge of the V3 loops at baseline ranged from +4 to +7. Similarly, only negatively charged or neutral amino acid residues were present at positions 11 and 25 in clones obtained during filgrastim treatment. The net charge of the V3 loop sequences from subjects 146, 147, 148, and 745 did not change during filgrastim treatment. However, 6 of 7 sequences in the unique phyletic subcluster obtained from subject 051 during filgrastim treatment (figure 1) had a net V3 loop charge of $\geq +7$, as compared to only 1 of 28 baseline sequences ($P < 0.001$; Fisher’s exact test).

DISCUSSION

Filgrastim commonly is used to treat neutropenic complications of HIV-1 disease and antiretroviral therapy and is occasionally used to mobilize HSCs in HIV-1–infected persons. Therefore, it is important to better understand the effects that filgrastim has on HIV-1 replication. The present study sought to determine whether HSC mobilization with filgrastim caused a general activation of HIV-1 replication or stimulated virus production in a subset of HIV-1–infected cells. Our analyses were limited to 7 subjects who had significant increases of plasma HIV-1 RNA (an increase above baseline of $\geq 0.6 \log_{10}$ copies/mL) during filgrastim treatment. In 2 of these 7 subjects, no change in the composition of the plasma quasi species was detected, whereas the composition of the plasma quasi species changed during filgrastim treatment in the remaining 5 subjects; in 3 of these 5 subjects, unique env genotypes emerged that produced substantial viremia (8,400–42,000 copies/mL) and that accounted for 47%–71% of the increase of plasma HIV-1 RNA in these subjects.

The findings of the present study differ from those of similar studies of the effects of generalized immune activation on the plasma quasi species. Treatment with IL-2 leads to stimulation of HIV-1 replication, without a change in the composition of the plasma quasi species [9], a pattern observed in 2 filgrastim-treated subjects in the present study; because only 10 env molecular clones per subject were analyzed, it is possible that small shifts in the plasma HIV-1 quasi species occurred in these 2 subjects but were not detected. In the other 5 subjects, the
effects that filgrastim had on the plasma HIV-1 quasi species more closely resembled the effects of immunization with recall antigens [18], which is thought to amplify minority viral variants by selective stimulation of discrete populations of HIV-1–infected cells.

Our findings do not suggest that filgrastim treatment activated a population of long-lived, latently HIV-1–infected cells in the subjects who had changes in plasma quasi-species composition. For the unique env genotypes that appeared in 3 subjects during filgrastim treatment, genetic distance from the shared MRCA was equal to or greater than that for the plasma quasi species at study entry. This finding suggests that the fil-
grastim-induced minor variants and the rest of the plasma quasi species evolved concomitantly. This interpretation is further supported by the finding that the virus present in the plasma of subject 051 at day 5 of filgrastim treatment contained PR mutations that conferred resistance to PR inhibitors. Because subject 051 was naive to the PR-inhibitor class 20 months before study entry, the finding of drug-resistance mutations in PR suggests that the minor variants that appeared during filgrastim treatment were actively evolving in response to the selective pressure of antiretroviral therapy.

It is likely that the selective pressure of the antiretroviral drugs that were taken during filgrastim treatment prevented the expansion of virus that lacked antiretroviral-drug–resistance mutations. Thus, although changes in the composition of the quasi species were observed when \( \text{env} \) sequence data were examined, filgrastim treatment had no effect on the composition of RT sequences in the plasma quasi species of subject 051. Compared with the plasma quasi species present just before filgrastim treatment, \( \text{env} \) of the unique virus induced by filgrastim treatment was more distantly related to the plasma quasi species present 5 years prior to study entry in subject 051. This finding, although anecdotal, suggests that the \( \text{env} \) sequences that constituted the majority of the quasi species at baseline were subjected to selective pressures different than those encountered by the \( \text{env} \) sequences that were selectively expanded by filgrastim treatment. Other than a slightly increased net positive charge in 1 of 3 subjects, there were no differences in predicted \( \text{env} \) phenotypes in the unique viruses. However, our analysis was limited to a relatively short, highly variable region of \( \text{env} \), and we did not evaluate the possibility that other regions of \( \text{env} \) could contribute to a distinct phenotype in these minority variants.

The mechanism by which HSC mobilization by filgrastim led to increased HIV-1 viremia is not known. Previous studies have not observed the effects that filgrastim—or other preparatons of G-CSF—might have on HIV-1 replication in vitro [19, 20]. However, the G-CSF receptor is present on both monocytes and activated T cells, and G-CSF augments monocyte-dependent differentiation and proliferation of T-cell lines [8, 21–23]. G-CSF also modulates cytokine production directly in monocytes and either directly or indirectly in lymphocytes [23–27]. Last, filgrastim partially restores defective IL-2 release from the blood cells of HIV-1–infected persons [28]. Thus, there are several plausible mechanisms by which filgrastim treatment could stimulate HIV-1 replication.

We propose the following model to explain the findings of the present study. Infected persons who have increased HIV-1 replication during filgrastim treatment have subsets of cells (CD4+ lymphocytes and/or monocytes) that are susceptible to both filgrastim stimulation and HIV-1 infection. In some cases, different selective pressures on \( \text{env} \) during HIV-1 replication in these cells results in the evolution of unique HIV-1 variants. In the absence of filgrastim stimulation, these cells normally do not make large contributions to the plasma quasi species. Filgrastim either directly or indirectly stimulates HIV-1 replication in this subset of host cells. When the filgrastim-sensitive cells are infected by unique HIV-1 variants, these variants are preferentially amplified by filgrastim stimulation. This, in turn, leads to a change in the composition of the plasma quasi species. Alternatively, if the filgrastim-stimulated cells contain viral variants that contribute to the majority plasma quasi species, then filgrastim stimulation causes expansion of the baseline quasi species. Further studies are necessary to define the phenotype of (1) the cells that are susceptible to both HIV-1 infection and filgrastim stimulation and (2) the virus that infects these cells.

We have previously reported that increased HIV-1 replication occurs during filgrastim treatment [10, 11]. A more recent study also found increased plasma HIV-1 RNA during filgrastim treatment of subjects who did not receive antiretroviral drugs, but not in subjects who received highly active antiretroviral therapy [29]. In both studies, increased HIV-1 replication was not associated with untoward effects, and plasma HIV-1 RNA returned to baseline values after 3 weeks after filgrastim treatment was discontinued. The present study found that unique HIV-1 variants appeared in some subjects during the period of increased HIV-1 replication that occurred during filgrastim treatment. Although these findings help us to understand how filgrastim affects HIV-1 replication, they do not provide additional information on the safety of filgrastim use in HIV-1–infected persons. Thus, the findings of the present study do not suggest that changes in filgrastim use in clinical care of HIV-1–infected persons are warranted.

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