Effect of a Protease Inhibitor-Induced Genetic Bottleneck on Human Immunodeficiency Virus Type 1 env Gene Populations

Kathryn M. Kitrinos,1,2† Julie A. E. Nelson,1‡ Wolfgang Resch,1,3§ and Ronald Swanstrom1,3*

UNC Center for AIDS Research,1 Curriculum of Genetics and Molecular Biology,2 and Department of Biochemistry and Biophysics,3 University of North Carolina at Chapel Hill, 22-062 Lineberger Cancer Center, CB 7295, Chapel Hill, North Carolina 27599-7295

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The initiation of drug therapy or the addition of a new drug to preexisting therapy can have a significant impact on human immunodeficiency virus type 1 (HIV-1) populations within a person. Drug therapy directed at reverse transcriptase and protease can result in dramatic decreases in virus load, causing a contraction in the virus population that represents a potential genetic bottleneck as a subset of virus with genomes carrying resistance mutations repopulate the host. While this bottleneck exerts an effect directly on the region that is being targeted by the drugs, it also affects other regions of the viral genome. We have applied heteroduplex tracking assays (HTA) specific to variable regions 1 and 2 (V1/V2) and variable region 3 (V3) of the HIV-1 env gene to analyze the effect of a genetic bottleneck created by the selection of resistance to ritonavir, a protease inhibitor. Subjects were classified into groups on the basis of the extent of the initial drop in virus load and the duration of virus load reduction. Subjects with a strong initial drop in virus load exhibited a loss of heterogeneity in the env region at virus load rebound; in contrast, subjects with a weak initial drop in virus load exhibited little to no loss of heterogeneity at virus load rebound in either region of env examined. The duration of virus load reduction also affected env populations. Subjects that had prolonged reductions exhibited slower population diversification and the appearance of new V1/V2 species after rebound. The longer reduction of virus load in these subjects may have allowed for improved immune system function, which in turn could have selected for new escape mutants. Subjects with rapid rebound quickly reequilibrated the entry env variants back into the resistant population. When the pro gene developed further resistance mutations subsequent to virus load rebound, no changes were observed in V1/V2 or V3 populations, suggesting that the high virus loads allowed the env populations to reequilibrate rapidly. The rapid equilibration of env variants during pro gene sequence transitions at high virus load suggests that recombination is active in defining the HIV-1 sequence population. Conversely, part of the success of suppressive antiviral therapy may be to limit the potential for evolution through recombination, which requires dually infected cells.

The development of antiretroviral therapy to treat human immunodeficiency type 1 (HIV-1)-infected individuals has had a significant impact on disease progression. While different combination therapy regimens (which typically include reverse transcriptase [RT] and protease [PR] inhibitors) have been shown to be effective initially in reducing plasma virus loads and increasing CD4+ T-cell counts, a significant percentage of individuals eventually fail therapy, as indicated by a rebound in plasma virus load (5, 12, 13, 29, 40). When effective combination therapy is used to treat subjects, the development of resistance can be delayed for years; however, when therapy is suboptimal, resistance can develop rapidly, often within weeks to a few months, and this is the typical outcome with monotherapy (reviewed in reference 38). The rebonding virus often has reduced susceptibility to the RT and/or PR inhibitors used in the treatment regimen. The virus load of this resistant virus can return to pretherapy levels; however, a subset of subjects have virus rebound to an intermediate level and may experience a sustained immunologic benefit as measured by a sustained increase in CD4+ T-cell levels (3, 4, 19, 31).

Antiviral therapy can result in a 100-fold or greater decrease in virus load even under conditions where the therapy is not sufficiently potent to prevent virus rebound. This transient contraction of the virus population represents a potential genetic bottleneck. There is some evidence that preexisting mutants with resistance-conferring mutations are present at low levels in the virus population and account for the appearance of resistant virus during rapid virologic failure (10, 15). Given an error-prone RT and high levels of replication, many point mutations should be maintained at some steady-state level if the effective population is large (reviewed in reference 2).

The effect of the drug-induced genetic bottleneck on the population dynamics of other regions of the viral genome has been addressed in several different studies (7, 9, 11, 17, 23, 28, 34, 36, 42). All of the studies have analyzed the effect of the drug-induced bottleneck on the gene encoding the viral surface glycoprotein (env), since it is the most variable region of the HIV-1 genome, often present as coexisting variants. Several different combinations of transiently suppressive therapy have...
been studied, such as RT inhibitors (9, 23, 34, 36, 42), protease inhibitors (7, 17, 28), or a combination of both (11). However, the results presented in these studies are conflicting.

There are several potential limitations in the experimental design of these studies that complicate a generalizable interpretation. First, in several studies the drop in virus load due to the initiation of therapy was either not shown or was less than 10-fold (9, 11, 23, 34, 36, 42), making it difficult to assess whether the drug regimen caused a genetic bottleneck. Second, in some of the studies the first sample collected after the initiation of therapy was 6 to 12 months later, making it difficult to determine if the env population changes were due to drug therapy or to selective pressures on env subsequent to rebound (34, 36, 42). Third, some studies followed subjects for only a few months and were unable to determine if the env population changes due to the bottleneck were transient (17, 19, 28). Fourth, the method used to analyze the samples in the majority of studies was direct cloning and sequencing, which can have limited sampling power to determine the population composition. One study by Delwart and colleagues (7) avoided these experimental limitations by employing a heteroduplex tracking assay (HTA) to examine closely spaced samples over approximately 5 months, and they were able to demonstrate that there was a transient change in env populations in one-half of the subjects during briefly suppressive therapy.

We have reexamined the question of a drug-induced bottleneck in closely spaced samples from subjects with various responses to therapy using a sensitive method for sampling the virus populations. We have developed HTAs specific for two different variable regions in env, variable regions 1 and 2 (V1/V2-HTA) (20), and variable region 3 (V3-HTA) (27), and used these assays to analyze env population dynamics in a group of subjects who added the protease inhibitor ritonavir to preexisting but failed nucleoside therapy. The HTA is a sensitive approach for studying virus population dynamics. Because it allows the visualization and quantitation of virus variants that comprise as little as 3% of the total population in a given sample, there is enhanced sampling potential, and the quality of sampling can be readily validated (6, 32). In our study 9 of the 10 subjects with a drug-induced but transient 100-fold decrease in virus load displayed a loss of heterogeneity from day 1 to rebound in at least one of the two env regions examined. In contrast, four subjects with less than a 100-fold decrease in virus load displayed minimal loss of env heterogeneity in spite of a turnover in the protease gene (pro) with the appearance of resistance-associated mutations. Subjects with a fast virus rebound exhibited a rapid diversification of V1/V2 variants with env variants present at baseline reemerging after rebound, whereas subjects with a slow virus rebound exhibited a delayed diversification of V1/V2 variants and had new env variants emerge after rebound. Although pro continued to accumulate resistance mutations after rebound, there was no evidence of further env population disruption, which implies rapid equilibration of virus populations at high virus load. Our data suggest that at high virus loads, recombination allows for the rapid equilibration of the virus population and that this mechanism of evolution may be reduced during suppressive therapy.

MATERIALS AND METHODS

Subject samples. Human plasma samples were obtained from Abbott Laboratories, Abbott Park, IL, from a placebo-controlled trial of ritonavir (M94-247) (1). All subjects were at the later stages of infection, as indicated by CD4+ T-cell counts below 100/μl. Subjects entering the trial continued their (failed) RT inhibitor regimens and added a twice-daily dose of oral ritonavir (600 mg). Blood plasma samples were collected every 2 to 4 weeks over approximately 9 months to a year.

Viral RNA isolation and RT-PCR. Viral RNA was extracted from 140 μl of blood plasma using the QIAamp viral RNA kit (QIAGEN) and recovered in 8 μl. V1/V2-specific RT-PCR was done as described previously (20) using the One Tube RT-PCR system (Roche Molecular Biochemicals). Briefly, RT reaction mixtures consisted of 5 μl of purified viral RNA, 1× Titan RT-PCR buffer, 5 mM dithiothreitol (DTT), 1 mM of each deoxynucleoside triphosphate (dNTP; Amersham Pharma), 15 pmol of primer V2R5, 10 U RNase inhibitor (Roche Molecular Biochemicals), and 12 U of avian myeloblastosis virus RT (AMV-RT) (Roche Molecular Biochemicals) in a total volume of 20 μl. Reaction mixtures were incubated at 42°C for 30 min, and then the AMV-RT was inactivated for 5 min at 95°C. An aliquot of 30 μl of PCR mixture (1× Titan RT-PCR buffer, 5 mM DTT, 15 pmol of primer V1, and 0.5 μl Titan enzyme mix) was then added to each reaction mixture. PCR was performed as described previously (20).

V3-specific RT-PCR was done as described previously with minor modifications (27). Briefly, RT reaction mixtures consisted of 5 μl of purified viral RNA, 1× Titan RT-PCR buffer (Roche Molecular Biochemicals), 2.5 mM MgCl2, 15 pmol of each dNTP, 15 pmol of primer V3R5, and 12 U of AMV-RT (Roche Molecular Biochemicals) in a total volume of 20 μl. Reaction mixtures were incubated at between 42°C and 45°C for 30 minutes, and then the AMV-RT was inactivated for 5 min at 95°C. An aliquot of 30 μl of PCR mixture (1× Expand Hf buffer, 2.5 mM MgCl2, 15 pmol of primer V3L4, and 2.6 U of Expand Hf enzyme mix [Roche Molecular Biochemicals]) was then added to each reaction mixture, and PCR was performed as described previously (20).

For all samples, duplicate PCR-RT amplifications and HTA analyses were performed to ensure adequate sampling. Sampling was validated by being able to reproduce both the number of detected HTA bands and the relative abundance of each band in the population. For most samples where the RNA copy number was less than 10^6/ml, twice as much RNA was used in the RT reaction. Alternatively, when possible, virions were pelleted from a larger plasma volume prior to RNA extraction. In general, we observed good sampling when the plasma viral RNA concentration is greater than 10^6/ml and in most of the cases where we detected a homogenous population, we were able to use RNA concentrations that were comparable to those where sampling of multiple variants could be validated. Finally, since we use a single round of PCR (i.e., not nested), the detection of a product after the single round further indicates significant template amounts were used to ensure good sampling.

V3-specific RT-PCR was performed using the Ba-L and JR-FL probes as described by Ketrinos et al. (20) with some minor modifications. Briefly, 6 μg of probe DNA was digested with NdeI. Probes were labeled in a fill-in reaction that included 10 mM DTT, 50 μM dTTP, 50 μCi of [35S]dATP (1,250 Ci/mmol; NEN Life Science Products), and 10 U Klenow fragment of DNA polymerase 1 at room temperature for 15 min, followed by heat inactivation of the enzyme at 75°C for 15 min. Unincorporated nucleotides were removed using a QIAquick PCR purification kit (QIAGEN). The probes were released from the vector by digestion with Kpnl, and then the reaction mixture was brought up to a final volume of 150 μl. Heteroduplex annealing reaction mixtures consisted of 1 μl 10× annealing buffer (1 M NaCl, 100 mM Tris-HCl [pH 7.5], 20 mM EDTA), 1 μl labeled probe, 0.1 μM each of V1 and V2 primer, and 8 μl unincorporated PCR product. Extra primers were added to the annealing reaction mixture to bind the unannealed probe and make its migration more uniform. Reaction mixtures were denatured at 95°C for 2 min, and then duplicates were allowed to form for 5 min at room temperature. Heteroduplexes were separated by electrophoresis in a 6% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) in 1× Tris-borate-EDTA (TBE) buffer, and then the gel was dried and analyzed by autoradiography or storage phosphor autoradiography (Molecular Dynamics). Previous work with the V1/V2-HTA has demonstrated that the sequence of a specific variant present in multiple samples from an infected person (i.e., one that migrates to the same position on the gel) has few, if any, undetected nucleotide changes between the samples, which established that distinct sequence variants most often migrate to different positions in the gel using V1/V2-HTA (20). The sequence differences that cause distinct variants to migrate to unique positions in the gel (defined as separated by at least one bandwidth) are clustered point mutations and size differences caused by insertions and/or deletions relative to the labeled probe.
The use of a heterologous probe enhances the ability of the HTA to resolve genotypic variants with only minor sequence differences, although not all variants are necessarily resolved by HTA. Conversely, the use of multiple different HTA probes increases the chance of resolving multiple variants (if they are present) with at least one of the probes.

**V3-HTA.** The V3-HTA was performed as described by Nelson et al. (27). Briefly, 1.5 μg of the JR-FL probe (27) or the clade C V3 probe (26) was digested with EcoRI. Probes were labeled in a fill-in reaction that included 25 μCi of [35S]dATP and 2 U Klenow fragment of DNA polymerase I at room temperature with EcoRI. Probes were labeled in a fill-in reaction that included 25 μCi of [35S]dATP and 2 U Klenow fragment of DNA polymerase I at room temperature with EcoRI. Probes were released from the vector by digestion with PstI, and unincorporated nucleotides were removed using a MicroSpin G-50 column (Amersham Pharmacia). The probe was brought up to a final volume of 100 μL. Heteroduplex annealing reactions consisted of 1 μL 10× annealing buffer, 1 μL labeled probe, 0.1 μM of primer V3L4, and 8 μL unpurified PCR product. Reaction mixtures were denatured at 95°C for 2 min, and then duplexes were allowed to form for 5 min at room temperature. Heteroduplexes were separated by electrophoresis in a 12% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide) in 1× Tris-borate–EDTA buffer. The gel was dried and then analyzed by autoradiography or storage phosphor autoradiography.

**Shannon entropy determination.** Shannon entropy was determined to analyze the complexity of bands in each subject’s virus population at different time points. The phosphorimage of each HTA gel was analyzed using ImageQuant, version 1.2 (Molecular Dynamics), and the fraction of the total volume each band represented within a time point was determined after subtracting the background volume. The entropy for each time point was then calculated using the following formula (8, 35):

$$H = -\sum_{i=1}^{n} p_i \log_2 p_i$$

(1)

where $p_i$ is the fraction of the total volume of band $i$ ($i = 1, 2, ..., n$, where $n$ is the total number of bands). The entropy is 0 if $n = 1$, and the entropy reaches its maximum value when all the bands within a sample are equally abundant.

**Divergence determination.** Divergence was calculated to analyze the fraction of the total population that contained newly emerged variants at time points subsequent to entry. The phosphorimage of each HTA gel was analyzed using ImageQuant, and the fraction of the total volume each band represented within a time point was determined after subtracting the background volume. The divergence for each time point was then calculated using the following formula:

$$D = 1 - \sum_{i=1}^{n} p_i$$

(2)

where $p_i$ is the fraction (determined at that time point) of the total volume of band $i$ ($i = 1, 2, ..., n$, where $n$ is the total number of bands that were present at day 1). If all the variants present at a particular time point were present at day 1 in the same proportion, then the divergence is 0. Alternately, if all of the variants present at a particular time point were different from those present at day 1, then the divergence is 1.

**Sequence analysis.** RT-PCR products of interest (both V1/V2 and V3) were purified using the QIAquick PCR purification kit (QIAGEN) and then cloned into the pT7Blue vector using the Perfectly Blunt cloning kit (Novagen). Individual colonies were screened by colony PCR, and the PCR products were analyzed by the relevant HTA. Plasmids containing variants of interest were prepared using the QIAprep Spin Miniprep kit (QIAGEN) and then sequenced using ABI Dye Terminator sequencing (Perkin-Elmer Corporation). Sequence analysis was performed using the Wisconsin Package, version 10.2, from the Genetics Computer Group.

**Nucleotide sequence accession number.** All sequences have been deposited in GenBank under accession numbers DQ113500 through DQ113595.

**RESULTS**

Characterization of virus populations at trial entry. A subset of 14 subjects infected with HIV-1 from the ritonavir arm of a clinical trial (1) were analyzed by V1/V2- and V3-HTA in order to assess the effect on env populations of a transient reduction in virus load caused by the selection for resistance in PR. The 14 subjects were selected based on distinctive patterns of response in virus load with the addition of ritonavir. All subjects were protease inhibitor naïve and had CD4+ T-cell counts below 100/μL and high virus loads in the blood (range = 4.57 to 5.79 log10; average = 5.35 log10 copies of viral RNA/mL of blood) at trial entry. Subjects continued any ongoing (failed) RT inhibitor therapy during the trial. Ten of the 14 subjects had changes in their RT inhibitor regimen during the 1-year trial, but only two subjects had significant alterations in their virus load or CD4+ T-cell counts. All subjects developed resistance to ritonavir as seen by using the MSS-HTA, which identified mutations in the pro gene whose identities were confirmed as resistance associated by sequence analysis (33). In addition, all subjects had only a transient response to the addition of ritonavir, with the virus load returning to or approaching pretreatment levels during the time period analyzed.

The 14 subjects were divided into three groups by their response to the addition of ritonavir. Two of the groups had a strong initial response to the therapy, as evidenced by at least a 100-fold drop in virus load. A group of short-term strong responders (1058, 1068, 1137, 1144, and 1147) had at least 100-fold drops in virus load after the addition of ritonavir but then returned to the entry value within 2 months (Fig. 1). A group of prolonged strong responders (1008, 1035, 1048, 1098, and 1118) had at least 100-fold drops in virus load after the addition of ritonavir but took longer than 2 months to return to the entry value (Fig. 2). A group of weak responders (1051, 1092, 1148, and 1157) had less than 100-fold drops in virus load, if any, after the addition of ritonavir (Fig. 3). The differences in response of the virus load to the initiation of therapy may reflect differences in drug exposure and the ability of the initial resistant variant to replicate in the presence of that level of drug (24).

Both V1/V2 and V3-HTAs were performed at the entry time point, the earliest rebound time point available, and several time points after rebound for all of the 14 subjects (Fig. 1 to 3). The V1/V2-HTA patterns were unique for each subject, with most subjects having multiple variants at trial entry (range = 1 to 7 variants; average = 3.5 variants, which did not vary between the strong and weak responders). These numbers are similar to those seen in subjects from the placebo arm of the same trial (20). The V3-HTA patterns were less complex than the V1/V2-HTA patterns, with 6 of 13 subjects having a single V3 variant and 7 subjects having two to three V3 variants at trial entry, again similar to the placebo arm of the trial (26).

**Loss of env heterogeneity in the V1/V2 region at virus rebound.** There was evidence of a significant contraction in the complexity of the env population at virus rebound in the two groups of strong responders compared to the weak responders. The rebound time point was defined as the point where the virus load started on an up-slope and/or when the first resistant pro variant emerged (time point with star in Fig. 1 to 3). One subject, 1048, could not be evaluated for a loss of heterogeneity in V1/V2 because we were unable to amplify this region from the viral RNA derived from this subject. Among the strong responders there was a clear reduction in the number of V1/V2 variants in comparing the entry sample to the rebound sample in subjects 1058, 1068, 1144, and 1147 (Fig. 1) and subjects 1098 and 1118 (Fig. 2). Thus, among the strong responders, six of nine showed a reduction in env complexity, with two others showing no change in the complexity (1008 and 1035; Fig. 2).
FIG. 1. V1/V2-HTA, V3-HTA, virus load graphs, and CD4⁺ T-cell count graphs for the five short-term strong responders. The time points on both gels are in days. The V1/V2-HTAs were performed with either the Ba-L probe (1068, 1137, and 1147) or the JR-FL probe (1058 and 1144). The single-stranded probe is denoted on the V1/V2-HTAs with an open circle on the right side of the gel. The V3-HTAs were performed with either the JR-FL probe (1068, 1137, 1144, and 1147) or the clade C probe (1058). The homoduplex is denoted on the V3-HTAs with a filled circle on the right side of the gel. The time point in both the V1/V2- and V3-HTA with a star denotes the rebound time point. The lines to the left of the V1/V2 and V3 gels denote the bands in the day 1 time points. The virus load is represented by the solid line with open squares, while the CD4⁺ T-cell count is represented by the dashed line with solid circles. The arrow with the star above it denotes the rebound time point; all other arrows denote time points where additional resistance mutations in pro were observed.
and one sustaining the low complexity present at entry (1137; Fig. 1). However, even when the complexity was maintained, the migration of the band frequently changed, indicating a new sequence variant (see below). In contrast, weak responders maintained more complexity through the rebound time point (subjects 1051, 1092, and 1157; Fig. 3) and were more likely to retain HTA bands similar to those present at entry (Fig. 3). The greater contraction in V1/V2 heterogeneity at virus
rebound in the strong responders versus the weak responders was reflected in the average number of variants present at rebound: 1.6 for strong responders versus 3.0 for weak responders. We observed a similar result when we evaluated the entropy, or the complexity, in the V1/V2 populations from trial entry to virus rebound. Six of the nine strong responders exhibited a decrease in entropy from trial entry to virus rebound, whereas only one of four weak responders exhibited a decrease in entropy that approached the value of the six in the strong responder group (Table 1). Mann-Whitney rank sum tests indicated a significant difference in entropy values at entry and rebound for the strong responders ($P = 0.02$), but not the weak responders. The lower percentage of subjects with a reduction of env variants in the weak responder group is correlated with the retention of high virus loads and not with the drug-induced bottleneck, since all subjects acquired resistance-associated pro mutations after the initiation of ritonavir therapy (33). These results suggest a rapid association of the resistance marker with multiple env variants in the weak responders, a process that is suppressed in the strong responders.

The greater contraction of V1/V2 heterogeneity in the strong responders compared to the weak responders also influenced the identity of the V1/V2 variants at virus rebound. Overall, 72% of the V1/V2 variants present at rebound for the
**TABLE 1. Loss of heterogeneity, predominant species present at and subsequent to rebound, and change in entropy in V1/V2 for the 14 subjects analyzed**

<table>
<thead>
<tr>
<th>Group and subject</th>
<th>At rebound</th>
<th>Subsequent to rebound</th>
<th>Change in entropy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change in no.</td>
<td>New variants</td>
<td>Entry variants</td>
</tr>
<tr>
<td>Strong responders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolonged response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1008</td>
<td>2–2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1035</td>
<td>3–3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1098</td>
<td>4–2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1118</td>
<td>5–1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Short-term response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1058</td>
<td>4–2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1068</td>
<td>7–2</td>
<td>1</td>
<td>1</td>
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<tr>
<td>1137</td>
<td>1–1</td>
<td>1</td>
<td>0</td>
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<tr>
<td>1144</td>
<td>2–1</td>
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</tr>
<tr>
<td>1147</td>
<td>3–1</td>
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<tr>
<td>Weak responders</td>
<td></td>
<td></td>
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<tr>
<td>1051</td>
<td>7–5</td>
<td>1</td>
<td>4</td>
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<td>1092</td>
<td>2–3</td>
<td>1</td>
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<tr>
<td>1148</td>
<td>1–1</td>
<td>0</td>
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<td>1157</td>
<td>4–3</td>
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</table>

Strong responders were new (or previously undetected), while only 25% of the rebound V1/V2 variants were new for the weak responders (Table 1). Therefore in the weak responders there was a tendency to retain the preexisting variants, while in the strong responders the contraction of the population was associated with the appearance of new (or previously undetected) variants.

A significant concern in observing a contraction in the complexity of the virus population is the potential for an artifact due to undersampling. In all cases where multiple variants were present, we were able to validate the sampling by reproducing the multiple variants with two independent RT-PCR amplifications. Single variants were detected in many cases at high virus loads. In a few cases, the presence of the single variant persisted during rebound to higher levels of virus load. Thus, we believe the contraction observed represents the real state of the virus population and not an artifact of poor sampling.

**Loss of env heterogeneity in V3 at virus rebound.** A similar trend of heterogeneity loss between the entry and rebound time points was detectable in the V3-HTA patterns, even though overall the complexity of the V3 population was less than the complexity of the V1/V2 population. For those subjects with a strong response to the initiation of ritonavir mono-therapy, 7 out of the 10 subjects (1008, 1035, 1058, 1098, 1118, 1137, and 1147) exhibited a loss of heterogeneity in V3 from two to three variants to one variant (Fig. 1). However, for the three subjects with a weak response to the initiation of ritonavir, only one subject, 1148, exhibited a loss of heterogeneity in the V3 region from two variants to one variant (Fig. 3). We were unable to amplify the V3 region for subject 1157. Thus, the majority of subjects in the two groups with a strong response to the initiation of ritonavir, only one subject, 1148, exhibited a loss of heterogeneity in the V3 region from two variants to one variant. We were unable to amplify the V3 region for subject 1157. Thus, the majority of subjects in the two groups with a strong response to the initiation of ritonavir, only one subject, 1148, exhibited a loss of heterogeneity in the V3 region from two variants to one variant. We were unable to amplify the V3 region for subject 1157. Thus, the majority of subjects in the two groups with a strong response to the initiation of ritonavir, only one subject, 1148, exhibited a loss of heterogeneity in the V3 region from two variants to one variant. We were unable to amplify the V3 region for subject 1157. Thus, the majority of subjects in the two groups with a strong response to the initiation of ritonavir, only one subject, 1148, exhibited a loss of heterogeneity in the V3 region from two variants to one variant.

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**Differences between short-term and prolonged strong responders in V1/V2 subsequent to rebound.** While the majority of subjects in the two groups with a strong response to ritonavir had a loss of V1/V2 heterogeneity at virus rebound, there were distinct differences subsequent to rebound between the group of subjects with a short-term response and rapid rebound in virus load versus the group of subjects with a prolonged response and slow rebound in virus load. One obvious difference was the tendency of the virus population in the short-term responders to rapidly diversify after rebound (1058, 1068, 1147; Fig. 1) while the prolonged responders were more likely to maintain a low-complexity population (1008, 1035, 1118; Fig. 2). The rate of V1/V2 rediversification was measured by the change in entropy from virus rebound to the end of the trial, which was approximately 9 months later. For four of the five subjects with a strong but short-term response, a more rapid rediversification of the V1/V2 population was observed, with a trend of entropy increasing from rebound to the end of the trial (Mann-Whitney rank sum test, \( P = 0.06 \)) (Table 1). This increase brought the entropy back up to the level seen at the entry time point for each of the four subjects (data not shown). For the four subjects with a prolonged response, diversification of the V1/V2 population was slower, with only one out of the four subjects having an increase in entropy from rebound to the end of the trial (Table 1). Therefore, the rate of rediversification of V1/V2 variants after virus rebound correlated with the rate of virus load rebound.

There was also a difference in the types of variants that represented V1/V2 diversification subsequent to rebound in the short-term versus the prolonged responders. For the five short-term responders, the V1/V2 variants that emerged subsequent to rebound were the same variants that were present...
at trial entry. This result is clearly seen in the V1/V2-HTAs for four of the five subjects (Fig. 1). The sequences of clones from time points immediately before and after rebound for two of the four subjects (1144 and 1147) confirmed that the variants that emerged subsequent to rebound were identical to those variants present at entry that had the same HTA mobility. Subject 1137 had a new V1/V2 variant emerge subsequent to rebound, but it differed from the trial entry variant by only two synonymous point mutations, revealing that the entry and rebound variants were the same at the amino acid level. This result can also be shown by calculating the divergence of the V1/V2 variants present at entry from the V1/V2 variants present at later time points (Fig. 4A). For four out of five short-term responders (1058, 1068, 1144, 1147), divergence from the V1/V2 variants present at entry at the time point after rebound did not exceed 0.2 and never exceeded 0.4 throughout the rest of the time courses. Thus for the short-term responders, the V1/V2 variants that represented the diversification subsequent to rebound were largely the same variants that were present at entry, and these results also demonstrate that after their return, these variants remained fairly stable throughout the rest of the time course. The sum of these results suggests that ritonavir monotherapy had a short, transient effect on the V1/V2 variant population in subjects with a strong but short-term response to ritonavir therapy.

In contrast, in the four subjects with a prolonged response to ritonavir the V1/V2 variants that emerged subsequent to rebound were different from those variants present at entry (Fig. 2). Three subjects (1008, 1035, and 1098) had new V1/V2 variants emerge at rebound, which then continued to evolve throughout the time course. Subject 1118 maintained an entry V1/V2 variant at rebound, which remained the predominant member of the population until 7 months after rebound, when new V1/V2 variants began to emerge. These results were confirmed by calculating divergence, which demonstrated that there was a complete turnover of V1/V2 variants at rebound and these remained distinct from the entry variants throughout the rest of the time course for subjects 1008, 1035, and 1098, whereas new V1/V2 variants did not occur for subject 1118 until 7 months after rebound (Fig. 4B). These results suggest that in subjects with a strong response and slow virus rebound, the genetic bottleneck caused by ritonavir monotherapy disrupted the V1/V2 variant population and gave rise to new V1/V2 variants.

Differences between short-term and prolonged strong responders in V3 subsequent to rebound. The V3 region had a similar trend as far as the types of variants that arose after virus rebound in the short-term and prolonged strong rebounders, even though the complexity of the V3 region was much less than that of the V1/V2 region. In the four short-term strong responders, two of the five subjects (1048, 1098) had new variants emerge subsequent to virus rebound, and one subject had a sustained shift in abundance between two preexisting variants (1035). The remaining two (1008, 1118) had a single variant that did not change until several months later (Fig. 2). Although the data are more limited, the V3 region follows a similar trend as the V1/V2 region in that the V3 population showed a greater and sustained disruption in the prolonged strong responders versus the short-term strong responders.

Changes in pro subsequent to rebound do not further affect env variants. The development of high-level ritonavir resistance does not occur with the initial rebound, but rather resistance mutations accumulate in a series of steps over time (10, 24, 33, 43). Accordingly, the initial resistant pro variants that emerged at rebound in these subjects continued to accumulate resistance mutations at different times throughout the rest of the trial (indicated by arrows on virus load graphs in Fig. 1 to 3) (33). However, the further accumulation of mutations in pro after rebound did not appear to have significant effects on the population distribution of env variants (Fig. 1 to 3). The V1/V2- and V3-HTA patterns displayed no differences when the amount of entropy at the 13 time points where further pro mutations emerged at least 2 months subsequent to rebound.

![Divergence graphs for V1/V2 variants in subjects with a short-term (A) or prolonged (B) strong response in virus load.](image-url)
were compared to time points immediately before and after (Mann-Whitney rank sum test, \( P = 0.4 \)). Thus, similar to the time of early changes in pro with the weak responders, the late changes in pro that occurred during high virus load did not measurably disrupt the env population in any of the groups of subjects.

**DISCUSSION**

In this study, we have made a correlation between the extent and duration of the reduction in virus load during transiently suppressive therapy and the extent of disruption in the env gene population. All subjects in this study had their virus populations pass through a genetic bottleneck, as evidenced by the evolution of resistance to the newly added protease inhibitor (33). However, the response of the env gene population varied dramatically depending on the extent and duration of the decrease in virus load. Thus the disruption of the env population is dependent on the extent of the change in virus load, not simply the passage through a drug-induced genetic bottleneck.

The effect of virus load on the env population can be described as two phenomena. One effect was observed at low levels of virus load, which was seen in two different settings. First, the groups of strong responders with a large drop in virus load had a contraction in the number of variants (Fig. 1 and 2) and in the entropy of the env gene population during rebound, with the appearance of pro gene resistance-associated mutations (33). The population contraction was also accompanied by the presence of new (or previously undetected) variants at rebound (Fig. 1 and 2). This pattern persisted past rebound in the subset of strong responders who had a delayed return to entry virus loads (prolonged responders), where there was a delayed rediversification of the env population and the establishment of new variants (Fig. 2 and 4).

A contrasting effect was seen at high virus load in three different settings. First, in the absence of a strong initial response to ritonavir (i.e., the weak responders), there was little or no disruption of the env population (Fig. 3), even though resistance mutations evolved in pro (33). Second, among the strong responders with a short-term response, there was a rapid rediversification of the env population subsequent to rebound (Fig. 1) with the reappearance of the env variants present at entry (Fig. 4). Third, for all groups the later appearance of additional resistance-associated mutations in pro at high virus load subsequent to rebound did not disrupt the env gene population (Fig. 1 to 3).

We propose that two biological phenomena are at work in changing the composition of the env gene population: one viral and one host (Fig. 5). The viral effect that may be involved is differing levels of viral recombination. We attribute the apparent lack of an impact of the drug-induced genetic bottleneck at high virus load to high rates of recombination that rapidly equilibrate the preexisting virus variants as resistance mutations become fixed. In this interpretation, we assume that a single drug resistance marker is passed (fixed) to the genomes with different env sequences by recombination. In this view the number of dually infected cells, a prerequisite for recombination, is sufficiently high to allow the spread of pro genes containing resistance mutations among the preexisting env gene variants. Conversely, at low virus load there is a contraction of the complexity of the virus population because of the reduced chance for recombination during the outgrowth of the genome(s) containing the resistance-associated mutations.

Recombination has been experimentally demonstrated in vivo (41) and can result in both large evolutionary jumps, as in the formation of intersubtype recombinant viruses (reviewed in reference 30), and in smaller evolutionary jumps, as in the recombinants that can form between closely related variants (14, 20, 25, 37). Furthermore, a study by Jung et al. (18) demonstrated that 75 to 80% of HIV-1-infected spleen cells contained at least two proviruses, showing that dually infected cells are common and thus fulfilling this criterion for recombination. HIV-1 readily carries out recombination between the two RNA copies of their dimeric genome (reviewed in reference 16), providing a robust mechanism for reassorting genetic variability.

The putative host effect that may be involved is a transient...
improvement in the host immune response during the time that the virus load is suppressed. The appearance of new variants at rebound after a strong response (≥100-fold drop in virus load in short-term responders and long-term responders; Fig. 5) could reflect improved selection against old env variants, presumably by antibodies. However, we cannot rule out stochastic events defining the env population by linkage to the pro resistance-associated mutations. The persistence of new variants during the prolonged, partially suppressive response may similarly reflect the continued suppression of the preexisting variants due to improvement of the immune response. Several studies have demonstrated that the initiation of fully suppressive drug therapy can lead to an initial increase in memory CD4 T cells followed by an increase in naïve CD4 T cells (21, 22, 31). Furthermore, it has been demonstrated in one study that viruses that emerge at rebound can evolve to become resistant to neutralization by autologous serum, in contrast to the viruses present at trial entry (39). Transiently suppressive therapy may give similar benefits and could account for the delayed diversification and the appearance of new variants seen in the prolonged strong responders.

The differences we observed between the three groups in our study may explain some of the conflicting observations of previous studies looking at the effect of drug therapy on env populations. First, the observed difference in env heterogeneity between subjects with either weak or strong initial responses to ritonavir are consistent with studies analyzing subjects with a maximum of a 10-fold drop in virus load (or unknown virus loads) where little or no change in env variants was observed (9, 11, 23, 34, 36, 42). Second, the reappearance of the entry env populations after virus rebound, even as pro goes through further genetic bottlenecks, may help to explain why some of the previous studies observed no differences in env populations after drug therapy, since they studied samples several months after virus load rebound (23, 34, 42). In a study by Delwart and colleagues (7), closely spaced samples were analyzed by V3/V5-HTA over a 5-month period in subjects with a 10- to 100-fold drop in virus load and one-half of the subjects were found to have changes in their env population at rebound. The subjects that had changes in their env population at rebound were associated with greater drops in their virus load and/or had their virus load drop to lower levels than those of subjects that did not exhibit env population changes at rebound, consistent with our observations.

In conclusion, we have observed the effects of a drug-induced genetic bottleneck with subjects having variable responses to antiviral therapy using samples that were closely spaced and applying a sensitive assay to analyze the virus population. The results of this study demonstrate that a stronger drop in the initial virus load and a longer duration of virus load drop after the initiation of therapy had an increased likelihood of reducing the complexity and altering the composition of the viral V1/V2 and V3 env populations. We propose that one of the benefits of suppressive therapy is the reduction of the potential for recombination, making the virus population a more stable target for both immune and drug selection. Our observation of rapid equilibrium of preexisting env variants with drug resistance markers in pro at high but not low virus load is consistent with this hypothesis.

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