Viral dynamics in human immunodeficiency virus type 1 infection

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The dynamics of HIV-1 replication in vivo are largely unknown yet they are critical to our understanding of disease pathogenesis. Experimental drugs that are potent inhibitors of viral replication can be used to show that the composite lifespan of plasma virus and virus-producing cells is remarkably short (half-life ~2 days). Almost complete replacement of wild-type virus in plasma by drug-resistant variants occurs after fourteen days. Indicating that HIV-1 viremia is sustained primarily by a dynamic process involving continuous rounds of de novo virus infection and replication and rapid cell turnover.

The natural history and pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection are linked closely to the replication of virus in vivo. Clinical stage is significantly associated with all measures of virus load, including infectious virus particles in blood, virus antigen levels in serum, and viral nucleic acid content of lymphoreticular tissues and peripheral blood mononuclear cells (PBMCs) and plasma (reviewed in ref. 18). Moreover, HIV-1 replication occurs preferentially and constitutively in lymphoreticular tissues (lymph node, spleen, gut-associated lymphoid cells, and macrophages)19-21; virus is detectable in the plasma of virtually all patients regardless of clinical stage22-26; and changes in plasma viral RNA levels predict the clinical benefit of antiretroviral therapy (R. Coombs, unpublished results). These findings emphasize the central role of viral replication in disease pathogenesis.

Despite the availability of probes to detect viral replication in HIV-1 disease, relatively little quantitative information is available regarding the kinetics of virus production and clearance in vivo, the frequency of virus and CD4+ cell population turnover and, the fixation rates of biologically relevant viral mutations23. This circumstance is largely due to the fact that previously available quantitative assays lacked sufficient potency to abrogate HIV-1 replication, and methods to quantify virus and determine its genetic complexity were not sufficiently sensitive or accurate. We overcame these obstacles by treating subjects with new investigational agents which partially inhibit the HIV-1 reverse transcriptase (zidovudine, NVP29) and protease (ABT-538, L-735,52430), by measuring viral load changes using sensitive new quantitative assays for plasma virus RNA14,15, and by quantifying changes in viral genotype and phenotype in uncultured plasma and PBMCs using automated DNA sequencing26 and an in situ assay of RT function37,38.

Virus production and clearance

Twenty-two HIV-1-infected subjects with CD4+ lymphocyte counts between 38 and 251 per mm^3 (mean 114, 102.5 cells per mm^3) were treated with ABT-538 (n = 10), L-735,524 (n = 8) or NVP (n = 4) in part of phase I/IIA clinical studies. The design and clinical findings of these trials will be reported elsewhere (K. Squires et al., and V.A.J. et al., manuscripts in preparation). Plasma viral RNA levels in the 22 subjects at baseline ranged from 10^6 to 10^7 molecules per ml (geometric mean of 10^6.6) and exhibited maximum declines generally within 2 to 6 weeks of initiating drug therapy (Figs. 1 and 2a). For ART-538 and L-735,524-treated patients, virus titers fell by as much as 10^7-fold (about decrease of 10^7-fold) whereas NVP-treated patients virus fell by as much as 10^5-fold (mean decrease of 10^5-fold). The overall kinetics of virus decline during the initial weeks of therapy with all three agents corresponded to an exponential decay process (Figs 1 and 2a).

The antiretroviral agents used in the study, despite their differing mechanisms of action, have a similar overall biological effect in that they block de novo infection of cells. Thus the rate of elimination of plasma virus that we measured following the initiation of therapy is currently determined by two factors: the clearance rate of plasma virus per se and the elimination (or suppression) rate of pre-existing virus-producing cells. To a good approximation, we can assume that virus-producing cells decline exponentially according to $s(t) = s(0) e^{-k_d t}$, where $s(t)$ denotes the concentration of virus-producing cells at time $t$ after the initiation of treatment and $k_d$ is the rate constant for the exponential decline. Similarly, we assume that free virus $v(t)$ is generated by virus-producing cells at the rate $s(t)k_d$ and declines exponentially with rate constant $k_d$. Then, for the overall decline of free virus, we obtain $v(0) = (s(0)k_d)^{-1} e^{-k_d t}$. The kinetics are largely determined by the slower of the two decay processes. As we have data only for the decline of free virus, and not for virus-producing cells, we cannot determine which of the two decay processes is rate-limiting. However, the half-life $t_{1/2}$ of neither process exceeds that of the combined.

With these considerations in mind, we estimated the elimination rate of plasma virus and of virus-producing cells by three different methods: (1) first-order kinetic analysis of that segment of the viral disappearance curve corresponding to the most rapid decline in plasma virus, generally somewhere between days 3 and 14, (7) fitting of a simple exponential decay curve to all viral RNA determinations between day 0 and the earlier or infection point (Fig. 1); and (3) fitting of a compounded decay curve
that takes into account the two separate processes of elimination of free virus and virus-producing cells, as described. Method (1) gives a $t_{1/2}$ of 3.8 ± 0.9 days; method (2) gives a $t_{1/2}$ of 3.0 ± 1.7 days; and method (3) gives a $t_{1/2}$ of 2.0 ± 0.9 days for the slower of the two decay processes and a very similar value, 1.8 ± 0.5 days, for the faster one. These are averages (± s.d.) for all 72 parents. Method (1) arguably provides the most complete assessment of the data, whereas method (2) provides a simpler interpretation (but slightly slower estimate) for virus decline because it fails to distinguish the initial delay in onset of antiviral activity due to the drug accumulation phase, and the time required for very infected cells to initiate virus expression, from the subsequent phase of exponential virus decline. There were no significant differences in the viral clearance rates in subjects treated with AIF-338, L-785,524 or NVP, and there was also no correlation between the rate of virus clearance from plasma and either baseline CD4+ lymphocyte count or baseline viral RNA level.

**Virus turnover**

Direct population sequencing. As an independent approach for determining virus turnover and clearance of infected cells, we quantified serial changes in viral genotype and phenotype with respect to drug resistance in the plasma and PBMCs of four subjects treated with NVP (Fig. 2). NVP potentially inhibits HIV-1 replication but selects for one or more resistant substitutions in the reverse transcriptase (RT) gene (PNAS). Two of these mutations result in dramatic decreases (up to 1,000-fold) in drug susceptibility and are associated with a corresponding loss of viral suppression in vivo. Genetic changes resulting in NVP resistance can then serve as a quantifiable molecular marker of virus turnover. A rapid decline in plasma viral RNA was
observed following the institution of NVP therapy and this was
associated with a reciprocal increase in CD4+ lymphocyte counts
(Fig. 3c and h). Both outcomes were of brief duration, return-
ing to baseline within 6-20 weeks in these four patients. The
proportion of virus in uncultured plasma and PBMCs that con-
tained NVP-resistance-conferring mutations (Fig. 2c) was deter-
mined by direct automated nucleotide sequencing of viral nucleic
acid (Fig. 2c) as previously described.11 We then validated this
method by reinfection experiments, confirming its sensitivity
for detecting RT mutants that comprise as little as 2% of the
reverse transcriptase population. Mutated mixtures of wild-type and resis-
tant HIV-1 RT cDNA clones (differing only at the second base
position of codon 190) were amplified and sequenced (Fig. 3c).
Varying proportions of wild-type and mutant viral sequences
were present in the original DNA samples (mutant composition: 0, 10, 30, 50, 70 and 100%) and were faithfully represented in the rela-
tive peak-to-peak heights (and in the relative peak-to-peak areas
of cytosine (C) and guanine (G)) residues at the second base
position within this codon. Bases of (cysteine) (mutant/wild type) nucleotide peak heights expressed in arbi-

trary fluorescence units were as follows (predicted/observed):
0/0; 10/18/4; 25/39/9; 50/49/3; 75/71/5; and 100/98/9.

We next determined the ability of direct population sequenc-
ing to quantify wild-type and mutant viral RNA genomes in clinical specimens. Figure 5b shows the sequence chromatograms of RT codons 179-190 from viruses directly from uncult-
ured plasma specimens of subject 1625 before (day -7) and after
(day +28 and +140) the initiation of NVP therapy. At day
+7, all codons within the amino-terminal half of the RT
gene (codons 1-250), including those shown, were wild-type at
positions associated with NVP resistance.11 However, after only
28 days of NVP therapy, the wild-type plasma virus popula-
tion was completely replaced by an NVP-resistant mutant popula-
tion differing from the wild-type at codon 190 (glycine/to-serine
substitution). After 140 days of drug therapy, this codon had
evolved further such that the plasma virus population consisted
of an equimixture of two drug-resistant strains. One containing
G190S and the other containing G190A. There were no other
NVP-resistance-conferring mutations detectable within the viral
RT gene.

FIG. 5 Quantitative detection of HIV-1 drug resistance mutations by
direct automated DNA sequencing. A DNA sequence chromatogram of RT
codon 190 from a defined mixture of wild-type (WT) and mutant/mult
HIV-1, cDNA clones differing only at the second base position of the
codon. Sequences shown were obtained from each mutant mixture
and are presented in the mRNA (non-coding) DNA strand. For example, the
mini-strand RTG sequence shown corresponds to the plus-strand
codon GGA (glycine). Similarly, the minus-strand TGTC sequence con-
sists of minus-strand codon CCA (serine), A, the single-letter amino-
acid code corresponds to the plus-strand DNA sequence. Mixed bases
containing a 50/50 wild type mutant are denoted as K.0. DNA sequence
chromatograms of RT codons 179-190 (data not shown) as the mini-
strand reference) derived from virus plasma were quantified by plotting
RTG before (day -7) and after (day +28 and +140) starting NVP
therapy. Codon changes resulting in amino-acid substitutions at position
190 were readily detected. For example, the plus-strand sequence at position
190 (day -7) corresponds to AGC (glycine), G, and the minus-strand sequence at position 190 (day +28) corresponds to ASC (serine, S) in the respective plus strands.

N190S 31% of the nucleotide motifs at wild-type and mutant cDNA clones (all) were pre-
tematically and the fact that the wild-type PCR amplifications were done
with 2,000 virus cDNA target molecules per reaction. HIV-1 RNA was
extracted from serum plated from uncultured plasma specimens (d), as described10. cDNA was prepared using Moloney murine leukaemia virus reverse transcriptase (GIBCO BRL) and an oligonucleotide primer
complementary to viral sequences 4,263 to 4,302 of the HXB2 sequence.22
The full-length viral reverse transcriptase gene (1,690 nucleotides) was ampli-
fied by means of a nested PCR using conditions and oligonucleotide primers
indicated in Figure 2 as previously reported.10 Subsequently
amplified fragments of the RT gene were also amplified using combinations
of the following oligonucleotide primers: (i) 5'-mG-2'-6,610, (ii) 5'-tG-2'-7,212,
(iii) 5'-G-2'-3,344, (iv) 5'-G-2'-3,346, (v) 5'-tG-2'-3,346, (vii) 5'-tG-2'-3,346, (viii) 5'-G-2'-3,346, (ix) 5'-tG-2'-3,346, and (x) 5'-G-2'-3,346.
All 5 primers incorporated the universal primer sequence for
kot:EQUI:mm:op primer sequence analysis. The HIV-1 only number is
many PCR reactions were determined (100,000 copies).
A total of these six expanded PCR amplifications of primary patient material
were done on each sample using different combination of primers, and
replicative-competent chemotypes are shown. Roughly, codon interpretation
was ambiguous. In the day -140 plasma sample from subject 1625
(200) of panel e), 0% complementarity (65%) strand could yield
Ag/AQG, GQG/GAQ, ATN/ATQ, AGA/AQG/GAQ or GQG/GAQ, in this case, we sequenced 7 fragment size
nucleotides and found that they encoded only serine or arginine. For se-

sequence, an automated ABI 373A sequenator and the Taa dye primer
Oligonucleotides (30,000) were used. Sequences were analyzed using a Sequencing (Gene Codes Corp) and Macrogen (Shkelman) software
Sequencer, and base pair positions were quantified using measuring against
peak-to-peak heights.
In all four subjects evaluated by direct viral population sequencing (Fig. 4), specific NVP-resistance-conferring mutations within the RT gene could be unambiguously identified and subsequently confirmed by molecular cloning, expansion and drug susceptibility testing. In all cases, mutant virus increased rapidly in the plasma and virtually replaced wild-type virus after only 2–4 weeks of NVP therapy (Fig. 2c). By analyzing the rate of accumulation of resistant mutant in the plasma population, we could obtain an independent estimate of the turnover rate of free virus. The rate of drug-resistant mutant virus is influenced substantially by the preexisting increase in the CD4+ cell population (which provides additional reservoirs for virus production) and therefore follows complex dynamics. However, we could obtain an estimate of these dynamics by making simplifying assumptions. We assume that wild-type virus declines exponentially with a decay rate $\alpha$, and that the drug-resistant mutant increases exponentially with the rate $\beta$. Thus, the ratio of mutant to wild-type virus increases exponentially at the combined rate $\alpha + \beta$. Our genetic RNA (cDNA) data allow us to estimate this sum. Knowing a constant loss in virus decline, we get $\beta = 0.037$, or a 33% daily virus production (average over 4 patients). Assuming that mutant virus rate exponentially, this corresponds to a doubling time of ~2 days, which is in excellent agreement with the measured elimination half-life of 2.0–0.9 days for plasma virus (Figs. 1 and 3). Turnover of viral DNA from wild-type to drug-resistant mutant in PBMCs was delayed and less complete compared to plasma virus, reaching levels of only 50–80% of the total PBMC-associated viral DNA population by week 20 (Fig. 2b). Measurement of the time required for resistant virus to spread in the PBMC population allowed us also to estimate the half-life of infected PBMCs. After complete turnover of mutant virus in the plasma pool, we may assume that PBMCs infected with wild-type virus decline exponentially at a rate $\alpha$, whereas cells infected by mutant virus are generated at a constant rate, but also decline exponentially at rate $\beta$. With these simplifying assumptions, the rate at which the frequency of resistant virus in the PBMCs increases in vivo is proportional to the estimate for the parameter $\beta$ and hence for the half-life of infected PBMCs. We obtained a half-life of ~25–100 days. This means that the average half-life of infected PBMCs is very long and of the same order of magnitude as the half-life of uninfected PBMCs$^{19,20}$. Based on the long half-life of PBMCs, and the fact that these cells harbor predominantly wild-type virus at a time (days 14–28) when most virus in plasma is mutant, we conclude that most PBMCs contribute comparatively little to plasma virus load. Instead, other cell populations, most probably in the lymphocytic$^+$ system$^{18,19}$, must be the major source of virus production.

Direct sequence analysis of viral nucleic acid revealed not only rapid initial turnover in viral populations but also consequential viral evolution with respect to drug resistance mutations. In subject 1625 (Fig. 4, top panel), wild-type virus in plasma completely replaced after 28 days of NVP therapy by mutant virus.

**Fig. 4 Quantitative detection of HIV-1 drug resistance mutations** in plasma and PBMC DNA by the in situ polymerase chain reaction (PCR) and Taqman PCR assays. Using a PCR primer and probe pair specific for a plaque isolate as described in Fig. 2, virus DNA was isolated from uninfected PBMCs and amplified as described in Fig. 3. The amplified DNA was then screened for resistance mutations in the reverse transcriptase and protease genes by hybridization to a 5' and/or 3' probe and analyzed as described. **TABLE**: HIV-1-1 DNA was prepared from viruses reisolated from uncultured plasma as described for Fig. 3. Viral DNA was isolated from cultured cell cultures and amplified as described in Table 1. The full-length viral reverse transcriptase and protease genes were amplified and analyzed as described in Table 2. The HIV-1-1 DNA was then screened for resistance mutations in the reverse transcriptase and protease genes by hybridization to a 5' and/or 3' probe and analyzed as described in Table 1. The full-length viral reverse transcriptase and protease genes were amplified and analyzed as described in Table 2.
TABLE 1: In viro functional analysis of HIV-1 RT clones

<table>
<thead>
<tr>
<th>Subject</th>
<th>Specimen</th>
<th>Functional clones</th>
<th>Non-resistant clones</th>
<th>Non-resistant clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1625</td>
<td>Plasma</td>
<td>day -7 80 80 (100%) 0 (0%)</td>
<td>18 27 (38%) 45 (82%)</td>
<td>14 77 (100%) 0 (0%)</td>
</tr>
<tr>
<td>1625</td>
<td>PBMC</td>
<td>7 83 183 (100%) 0 (0%)</td>
<td>14 121 (100%) 0 (0%)</td>
<td>7 83 (100%) 0 (0%)</td>
</tr>
<tr>
<td>1625</td>
<td>PBMC</td>
<td>7 83 183 (100%) 0 (0%)</td>
<td>14 121 (100%) 0 (0%)</td>
<td>7 83 (100%) 0 (0%)</td>
</tr>
<tr>
<td>1624</td>
<td>Plasma</td>
<td>7 19 19 (100%) 0 (0%)</td>
<td>14 34 (12%) 30 (88%)</td>
<td>12 9 (100%) 0 (0%)</td>
</tr>
<tr>
<td>1624</td>
<td>PBMC</td>
<td>24 24 (100%) 0 (0%)</td>
<td>14 24 (100%) 0 (0%)</td>
<td>24 24 (100%) 0 (0%)</td>
</tr>
<tr>
<td>1625</td>
<td>PBMC</td>
<td>14 29 (80%) 21 (20%)</td>
<td>14 34 (12%) 30 (88%)</td>
<td>29 6 (8%) 22 (92%)</td>
</tr>
<tr>
<td>1625</td>
<td>PBMC</td>
<td>25 29 (90%) 3 (10%)</td>
<td>25 29 (90%) 3 (10%)</td>
<td>25 29 (90%) 3 (10%)</td>
</tr>
<tr>
<td>1610</td>
<td>PBMC</td>
<td>31 11 (70%) 20 (30%)</td>
<td>31 11 (70%) 20 (30%)</td>
<td>31 11 (70%) 20 (30%)</td>
</tr>
<tr>
<td>1610</td>
<td>PBMC</td>
<td>41 0 (0%) 41 (100%)</td>
<td>41 0 (0%) 41 (100%)</td>
<td>41 0 (0%) 41 (100%)</td>
</tr>
</tbody>
</table>

Full-length RT genes were analyzed by PCR from uncloned plasma and uncloned PBMCs as described in Fig. 3 legend. DNA products were cloned into the EcoRI and HindIII sites of the bacteriophage plasmid M13 [18] (with 26-28). The expression plasmids were screened by the presence of functional RT as tested in vitro for susceptibility to PRI inhibition at 100 nM of PRI and for CAL-replicon in the Vero cell system. Each cloned RT gene was then tested by sequential, non-competitive, reverse transcriptase (RT) assay performed in triplicate. The reverse transcriptase activity was then determined from the rate of reaction determined by primer extension reaction. The rate of reaction is expressed as pmoles of product formed per min per mg of protein. The reverse transcriptase activity was determined in the presence of 1 mM dNTPs and 100 nM template/primer. The reverse transcriptase activity was determined in the presence of 1 mM dNTPs and 100 nM template/primer.

C411 lymphocyte changes

Changes in C411 lymphocyte counts during the first 28 days of therapy could be assessed in 17 of our patients (Fig. 2a and Table 1). C411 cell number increased in every patient by between 41 and 830 cells/mm³ for the entire group, the average increase was 186 ± 190 cells/mm³ (mean ± S.D.) and 268±1399 from baseline. As C411 lymphocytes increase in number, so do the absolute number of peripheral blood monocytes, and the absolute number of CD3⁺ CD8⁺ cells, which increase in proportion to the total lymphocyte count. The increase in CD411 lymphocyte count is due to an increase in CD411 T cells, which are the major source of C411 lymphocytes. The CD411 lymphocyte count is inversely correlated with the CD8⁺ T cell count. The CD411 lymphocyte count is inversely correlated with the CD8⁺ T cell count. The CD411 lymphocyte count is inversely correlated with the CD8⁺ T cell count.
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explained by a unique or uncommon drug effect or a peculiarity of any particular virological assay method. Moreover, when new cycle of infection is interrupted by potent antiviral therapy, plasma virus levels abruptly drop by about 99%, and in some cases by as much as 99.9% (10,000-fold). This result indicates that the vast majority of circulating virus particles derives from continuous rounds of de novo virus infection, replication and cell turnover, and not from cells that produce virus in which the virus is replication-competent. The identity and location of this actively replicating cell population is not known, but appear to reside in the PBMC pool, consistent with prior reports (3,12). Nevertheless, PBMCs traffic through secondary lymphoid organs and to some extent are in equilibrium with these cells (13). It is thus possible that a small fraction of PBMCs (14,15) could make an important contribution to virusha.

The magnitude of ongoing virus infection and production required to sustain steady-state levels of viremia is extraordinary—based on a virus life span of 2.0 days and first-order clearance kinetics (c(t) = e^(-kt)), when t = 0.905(1/k), 30% or more of the total virus population in plasma must be replenished daily. For a typical HIV-1-infected individual with a plasma virus titer equal to the prereatment geometric mean in this study (10^6 RNA molecules per m1/2 RNA molecules per virus = 10^10 viruses per ml) and a plasma volume of 3 liters, this amounts to (4 X 10^10) [(1 X 10^10)/3] = 1.3 X 10^10 virus per day (for all 12 subjects, 2 X 10^10 x 10, 8x). Even if this may be a substantial underestimate of virus production because virions may be inefficiently transported from the intestinal extrafollicular spaces into the plasma compartment and viral protein expression assay (slope of mature particle formation) may result in cytopathy or immune-mediated destruction. Because we have shown that cells producing the majority of plasma virus cannot exceed 30 days, about 30% of these cells must be replaced daily. In our patients, we estimated the rate of CD4+ lymphocyte turnover to be, or average, 2 X 10^7 cells per day. or about 5% of the total CD4+ lymphocyte population, depending on clinical stage. This rapid ongoing recruitment of CD4+ cells into a short-lived virus circuitry or in rationally defined in vitro or in vivo CEM lymphocyte numbers that are observed immediately following the initiation of potent antiretroviral therapy, and suggests the possibility of successful immunological reconstitution even in late-stage disease if effective control of viral replication can be sustained.

The kinetics of virem+ and CD4+ lymphocyte production and clearance reported here have a number of biological and clinical implications. First, they are indicative of a dynamic process, involving continuous rounds of de novo virus infection, replication and rapid cell turnover—thus probably represents a primary driving force underlying HIV-1 pathogenesis. Second, the demonstration of rapid and virtually complete replacement of wild-type virus by drug-resistant virus in plasma after only 14-28 days of drug therapy is a striking example of the capacity of the virus for biologically relevant change. In particular, this implies that HIV-1 must have enormous potential to evolve in response to selection pressures as exerted by the immune system. Although other studies (16) have provided some evidence that virus turnover occurs sooner in plasma than in PBMCs, our data show this phenomenon most clearly. A similar experimental approach involving the genetic and phenotypic analysis of plasma virus could be helpful in identifying viral mutations and selection pressures involved in resistance to other drugs, immune surveillance and viral pathology. Third, the difference in lifespan between virus-producing cells and latently infected cells (PBMCs) suggests that virus expression per se is directly involved in CD4+ cell destruction. The data do not sug-

gest an "innocent bystander" mechanism of cell killing whereby uninfected or latently infected cells are indirectly targeted for destruction by adsorption of viral protein or by auto-immune activities.

Although we have emphasized that most virus in plasma derives from an actively replicating short-lived population of cells, latently infected cells that become activated or chronically producing cells that generate disproportionately low virus (and thus do not contribute substantially to the plasma virus pool) may nonetheless be important in HIV-1 pathogenesis. Based on the virus analysis (17), these cells for outnumber the actively replicating pool and the diversity of their constituent viral genomes represents a potentially important source of clinically relevant variants, including those conferring drug resistance. In future studies, it will be important not only to detect the specific elimi-
nation rates of live virus and of the most actively producing cells, but also the dynamics of virus replication and cell turnover in other cell populations and in patients at earlier stages of infec-
tion. Such information may contribute to developing a better understanding of HIV-1 pathogenesis and a more rational approach to therapeutic intervention.