Evolution of HIV-1 quasispecies and coreceptor use in cell reservoirs of patients on suppressive antiretroviral therapy

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Objectives: To track changes in the V3 env region of HIV-1 quasispecies and determine virus coreceptor use in cell reservoirs of patients on long-term suppressive antiretroviral therapy (ART).

Patients and methods: Ten patients whose plasma viremia had been suppressed for a median of 5.5 years were followed for 5 years. The V3 env regions of viruses in peripheral blood mononuclear cells were analysed by ultra-deep sequencing (UDS). HIV-1 tropism was predicted using the geno2pheno 5.75 algorithm and a phenotypic assay.

Results: The UDS and phenotypic assay data were concordant for predicting HIV-1 tropism. CXCR4-using viruses detected by UDS accounted for 14.7%–76.5% of the virus populations in samples from five patients at enrolment. Five patients harboured pure R5 virus populations and no X4 viruses emerged during the 5 years. The selection pressures estimated by the dN/dS ratio were acting on the V3 region to produce diversification of the quasispecies in CXCR4-infected patients and purification of the quasispecies in R5-infected patients on effective ART.

Conclusions: UDS showed that the virus quasispecies in cell reservoirs of patients on long-term suppressive ART continued to evolve. CXCR4-using variants became more diversified. Analysis of the selection pressures on the virus quasispecies could provide a clearer picture of virus persistence in patients on effective ART.

Keywords: V3 envelope region, ultra-deep sequencing, selection pressure

Introduction

Highly active antiretroviral therapy (ART) decreases the plasma load of HIV-1 RNA to below the detection limit of standard assays, resulting in dramatic improvements in the clinical course of HIV-1 infections. However, the HIV-1 that persists in cellular reservoirs prevents eradication of the virus. Assays of HIV-1 RNA in peripheral blood mononuclear cells (PBMCs) have shown changes in DNA sequences even in patients on prolonged effective ART. The env gene of HIV-1 is subject to high selection pressure so that its sequence varies greatly. This region is important because it is responsible for the capacity of HIV-1 to use coreceptors for entry into host cells. CCR5-using viruses are classified as R5 variants, CXCR4-using viruses as X4 variants, viruses that use both coreceptors as R5X4 dual-tropic variants, and mixtures of R5, X4 and/or R5X4 variants as R5X4 dual-mixed.

New genotypic methods allow clonal analysis of the virus quasispecies with description and quantification of the minor variants. We therefore used ultra-deep sequencing (UDS) to investigate the temporal evolution of HIV-1 V3 sequences in 10 patients with sustained suppression of plasma HIV viremia who had been on potent antiretroviral treatment for a median period of 10 years.

Patients and improvements

The 10 patients enrolled were infected with subtype B HIV-1 and had had sustained optimal virological responses to ART for a median of 5.5 years (Table 1). They were treated at the Department of Infectious Diseases of Toulouse University Hospital, France, and were participants in the ANRS EP32 study. The median age of the patients was 47 years (IQR, 42–52) and their plasma HIV-1 RNA remained undetectable for a further 5 years (<40 copies/mL measured every 3–6 months by the COBAS® AMPLICOR HIV-1 Monitor test, Roche Diagnostics). The optimal virological response was defined as having a viral load <40 copies/mL at each measurement, tolerating only one blip in viral load (<200 copies/mL) throughout the study. No patient received coreceptor antagonists. This research was approved by the Institutional Review Board of Toulouse University Hospital and the patients gave their informed consent. HIV-1 tropism...
was determined at enrolment using the TTT recombinant phenotypic assay.8 Briefly, a gene fragment encompassing the gp120 and the ectodomain of gp41 of HIV-1 was extracted from PBMCs and amplified by PCR and nested PCR. The phenotype of HIV-1 coreceptor usage was determined using a recombinant virus entry assay with infection of U87 indicator cells bearing CD4 and either CCR5 or CXCR4. CXCR4-using viruses were detected when they accounted for ≥0.5% of the total population.

UDS was performed on the 454 GS Junior system. The two PBMC samples (taken after 5.5 and 10 years of effective ART) stored for each patient were tested in the same run. A 415 nt fragment encompassing the V3 env region was generated by nested PCR as previously described.9 Pooled PCR products were clonally amplified on capture beads in water-in-oil emulsion micro-reactors. A total of 500000 DNA-enriched beads were deposited in the wells of a full GS Junior Titanium PicoTiterPlate device and pyrosequenced in both forward and reverse directions. The sequence reads of the V3 env regions were quantified with GS AWA software version 2.5p1 (Roche Diagnostics) and aligned with the Bol (GenBank AY426110) consensus sequence, and the alignments were edited manually to correct for insertions or deletions in homopolymeric regions. The tropism of each virus clone was inferred from the V3 amino acid sequence by geno2pheno 5.75 available at http://coreceptor.bioinf.mpi-inf.mpg.de/index.php.10 The frequency of errors resulting from V3 amplification and deep sequencing was assessed by analysing the pyrosequencing data from a panel of 10 plasmid clones of env as described previously.7 The mean error rate of pyrosequencing was 0.031% (95% CI, 0.005%–0.056%). The detection threshold of minor X4 variants is indicated as a function of the number of reads of V3 for each sample. The rates of synonymous substitutions per synonymous site (dS) and non-synonymous substitutions per non-synonymous site (dN) were calculated by the method of Nei and Gojobori with the Jukes–Cantor correction11 of the strength of the positive (r > 0.13). The dN/dS ratio is an indicator of the number of unique amino acid substitutions or deletions in homopolymeric regions. The number of unique amino acid substitutions or deletions in homopolymeric regions. The number of unique amino acid substitutions or deletions in homopolymeric regions. The number of unique amino acid substitutions or deletions in homopolymeric regions. The number of unique amino acid substitutions or deletions in homopolymeric regions.

### Results

At the time of enrolment, the patients had been on effective treatment for a median of 5.5 years and the TTT phenotypic assay indicated the presence of R5X4 dual-mixed virus populations in five patients and pure R5 virus populations in the other five. UDS detected CXCR4-using viruses that accounted for 14.7%–76.5% of the virus populations in the five R5X4 phenotyped samples (Table 1). The R5X4 quasispecies contained more unique clones (median 19) than did the R5 quasispecies (median 7; P = 0.027). The correlation between the proportion of CXCR4-using viruses and the number of unique clones in the samples was positive (r = 0.696, P = 0.025 in Spearman’s rank correlation test). Five years later, the R5-infected patients still harboured only CCR5-using clones. The R5X4 quasispecies still had more unique clones (median 15) than the R5 quasispecies (median 10) but the difference was no longer significant (P = 0.13). The mean proportion of CXCR4-using clones in patients infected with R5X4 dual-mixed viruses increased from 54% to 60.7% but the difference was not significant (P = 0.63). The number of unique amino acid clones in the R5-infected patients tended to increase (P = 0.0975), whereas it was stable in the R5X4-infected patients (P = 0.86). The cell-associated HIV DNA content was stable in the patients under effective highly active ART between the two times of observation (mean, 2.15 log copies/10^6 PBMCs, data not shown).

We used the sequences obtained by UDS to construct phylogenetic trees by the neighbour-joining method, which showed no
clustering with respect to sample timing or tropism. Phylogenetic analysis excluded any possibility of sample contamination (data not shown). We evaluated the selection pressure on the V3 region of the quasispecies using the dN/dS ratio (Figure 1). The medians of the intrasample dN/dS ratios at enrolment were 1.23 for patients with CXCR4-using variants and 0.53 for those with only CCR5-using variants ($P = 0.059$). The medians of intrasample dN/dS ratios 5 years later were 1.58 for patients with CXCR4-using viruses and 1.05 for those with only CCR5-using variants ($P = 0.55$). The median of the interstage dN/dS ratio for patients infected with CXCR4-using variants (1.39) differed from that for patients with exclusive CCR5-using variants (0.66; $P = 0.075$).

**Discussion**

We used UDS to investigate genetic evolution in the V3 env region of HIV-1 quasispecies in 10 patients who had been given effective ART for a median duration of 10 years. While we found no tropism switching in individual patients during the 5 years, analysis of sequences generated by UDS showed that selection pressures had different impacts on the R5 and R5X4 virus populations in PBMCs.

The evolution of virus tropism during suppressive ART is controversial. Three studies reported <10% of tropism switching from R5 to X4 in patients on ART for 2–3.5 years, whereas two other studies described 27%–48% of tropism switching in a period of 1–5 years. These discrepancies could be due to the heterogeneity of the nadirs of CD4+ T cell numbers, which varied widely between patients and were correlated with the frequency of tropism switching. Moreover, one study included only treatment-naive patients, while others included previously treated patients. Our patients had been on effective ART for a median of 5.5 years, unlike those included in previous studies. Thus, we examined the virus quasispecies in patients with long-term
virological suppression. The virus DNA in PBMCs was submitted to selection pressures, but no tropism switch occurred.

UDS detected sequence variations in the V3 region of the virus DNA despite plasma HIV-1 RNA being undetectable. The V3 regions of the virus quasispecies in PBMCs were modified, as shown by the substitution rates and the interstage dN/dS ratios, which assess selection pressure over time. Intrasample dN/dS ratios in the pure R5-infected patients indicated a change from purifying to neutral selection during the 5 year interval. The median of the interstage dN/dS ratio indicated purification of the V3 quasispecies in patients infected exclusively with CCR5-using viruses. The nature of the selection pressures on these sequences is not known. Conventional clonal analysis previously indicated a reduction in genetic diversity in the V3 region of the virus population in patients on effective ART.

These observations on virus persistence may be considered potentially useful implications for the mechanisms responsible for virus persistence. Further investigation is needed to establish whether these results can be reproduced in a larger number of infected patients and whether virus persistence under ART is associated with specific patient or viral characteristics.

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Transparency declarations
None to declare.