Clade B HIV-1 superinfection with wild-type virus after primary infection with drug-resistant clade B virus

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**Background:** The immunological response to HIV-1 infection has been postulated to impede superinfection with a second virus; however, a few recent reports have documented cases of HIV-1 superinfection in humans either from different viral clades or from the same clade.

**Objective:** To differentiate between co-infection and superinfection in a patient harboring a distinct wild-type HIV 4 months after primary infection with drug-resistant HIV.

**Methods:** Detailed dye primer and clonal sequencing along with length polymorphism analysis was used to investigate the evolutionary linkage between viral populations sampled at different timepoints.

**Results:** After a set point viral load of ~6000 copies HIV RNA/ml, the viral load jumped to 34,000 copies/ml at month 4 and, shortly after, to almost 200,000 copies/ml. At that time a second viral strain was first detected by dye primer sequencing of a pol fragment. These findings were confirmed by analysis of a 1300 bp gag–pol fragment and clonal sequencing and phylogenetic analysis of the V3 region. Length polymorphism analysis of the gp120 V4–V5 region showed that the second viral population was absent even as a minority population until month 4, when it was found to be the majority population, and the initial variant was present only as a minority. Both strains were subtype B.

**Conclusion:** These data support intraclade HIV-1 superinfection by wild-type virus in the absence of antiretroviral therapy in a patient initially infected with drug-resistant HIV. The substantially different in-vivo viral growth characteristics observed illustrate the potential for superinfection to impact disease progression.
Introduction

The immunological response established during primary HIV-1 infection results in partial control of the infecting virus [1–3], and has been proposed to limit subsequent re-infection [4]. However, genetic mosaics resulting from interclade recombination events provide evidence for superinfection events in vivo. The occurrence of such events has been recognized in sub-Saharan Africa, Asia and South America, where divergent subtypes co-circulate [5–8]. Recombination is more readily discerned after co-infection with virus from different clades. The isolation of such recombines does not, however, always identify the individual who was infected and does not discriminate co-incident double infection from superinfection.

Two recent publications directly demonstrated superinfection by viruses from a clade different from that of the initial infecting strain [9,10]. Under these circumstances, clade-specific immune responses appear to have been insufficient to prevent infection by the divergent second strain. Here we report a case of superinfection with virus of the same subtype as the initial infecting viral strain, occurring in an untreated patient during the first year of HIV infection. In another recently published report [11], same-clade superinfection occurred in a patient during structured treatment interruption. The occurrence of intra-subtype superinfection extends concerns about superinfection to those populations infected by a single predominant viral subtype, and has additional implications for vaccine development and clinical management.

This patient was one of a cohort of patients with transmitted drug resistance discovered during primary infection [12]. Upon enrollment, he had a CD4 cell count of 711 cells/µl and low plasma viremia, with 2413 copies/ml approximately 4 weeks after infection, and had received no antiretroviral treatment. Serial plasma and peripheral blood mononuclear cell (PBMC) samples were studied by dye primer sequencing of a 500 base pair (bp) fragment of pol in an attempt to characterize the reversion of drug resistance. At month 4, a second, apparently distinct, viral population was noted (data not shown). To confirm the presence of two diverse viral strains we performed sequencing of a 1302 bp fragment of the gag–pol region from nine plasma samples taken over the course of 12 months after primary infection. This showed a second viral strain that was, in contrast to the first strain, wild type at codons 181 and 184 of HIV reverse transcriptase, and showed additional polymorphisms (K20R, M36I, L63P) in protease that were not present in the first strain.

Phylogenetic analysis showed clustering of the three sequences at baseline, months 1 and 3 after infection, which segregated from the cluster of six sequences derived from plasma samples after month 4 (Fig. 1). Genetic distances (DNADIST version 3.5c, J. Felsenstein, University of Washington, Washington, USA) between pol sequences from early (patient group 1) and late timepoints (patient group 2) averaged 6.5%, and were similar in magnitude to the genetic distances between epidemiologically unlinked patients with primary HIV infection in San Diego (mean interpatient distance 5.6%). Both early and late viral variants belonged to clade B subtype, consistent with the epidemiological history from this patient (Fig. 1).

Clonal sequencing of the V3 env region was then conducted using 20 clones from two timepoints before and two timepoints after the first detection of a second viral strain. Phylogenetic reconstruction of these clonal sequences again demonstrated two distinct viral clusters. However, we could not detect a mixed population either before or after month 4, suggesting that the viral populations at both early and late timepoints contained less than 5% of the other virus (not shown). To differentiate further between superinfection and co-

Fig. 1. Inferred maximum likelihood phylogeny of pol sequences from the study patient before (patient group 1, sequences from three timepoints), and 4 months to one year after infection (patient group 2, sequences from six timepoints), sequences from 60 unlinked San Diego area patients, prototypic subtypes A–G, and HIV LAI. All sequences from the study patient were subtype B, but sequences obtained before 4 months clustered separately from those obtained between months 4 and 12. All San Diego reference sequences (unlabelled branches) but one (A U455 ACC) clustered as subtype B. Analysis performed with FastDNAML (Gary Olsen, University of Illinois, Illinois, USA) adapted from DNAML (Joseph Felsenstein, University of Washington, Washington, USA). Phylogenetic reconstruction by neighbor joining methods with bootstrapping produced similar results and high bootstrap values for independent clustering of the two patient viral groups.
infection we conducted a detailed analysis of length polymorphisms of the V4–V5 region using Genescan technology, a method with greater sensitivity for detecting minor populations. In the first 3 months after infection, plasma virus comprised a homogeneous viral population of uniform length (Fig. 2a). After month 4, a new length variant, three codons longer than the original infecting strain was detected (Fig. 2b). In one of two replicate genescan experiments at month 4 we were able to detect both the earlier virus as a minority population together with the new, dominant and longer variant (Fig. 2c). These length polymorphisms were confirmed by direct sequencing of the V4–V5 region from timepoints before and after superinfection occurred (data not shown).

HIV viral load alterations occurred over the period of observation. At baseline and up to month 3 the patient’s plasma viral load was relatively low, with only 2413 and 7148 viral copies, respectively. Coinciding with the detection of the second viral strain, the patient showed an abrupt and steep increase in his plasma viremia to 34,119 copies at month 4 and 199,686 and 170,712 copies at months 6 and 9, and a concurrent decline in the CD4 cell count from 792 cells/µl before superinfection to 328 cells/µl at month 12 after infection (Fig. 3). The lowest CD4 cell count of 283 cells/µl 11 months post-infection.

Materials and methods

The study was performed with appropriate patient consent. The gag-pol sequences have been submitted to GenBank as AY197730–AY197738.

RNA and DNA purification

RNA and DNA purification was performed on patient plasma and PBMC from a total of nine timepoints over a course of 12 months after primary infection using the Qiamp Viral RNA extraction kit and DNA kits (Qiagen, Chatsworth, CA, USA), respectively, according to the manufacturer’s instructions. All RNA and DNA extractions and subsequent amplification reactions were carried out with appropriate negative controls in parallel to detect contamination at each step of the procedures.

Plasma HIV-RNA assays

Plasma RNA was assayed using the Roche Amplicor Ultrasensitive assay (limit of detection 50 copies/ml).
Reverse transcriptase–polymerase chain reaction

Reverse transcriptase–polymerase chain reaction (RT–PCR) of plasma-derived RNA was performed using the Finnyzme Robust RT–PCR kit (MJ Research, Waltham, MA, USA). RT–PCR was performed in a nested manner using the following primers: RTF-1c, RTB-1c as the outer primers, cycling parameters: 48°C for 45 min, 95°C for 2 min, 95°C for 30 s, −50°C for 1 min, −72°C for 1 min for 35 cycles, 72°C for 10 min, followed by a second amplification using M13F-5RT, M13R-3RT primers at 95°C for 2 min, 95°C for 30 s, −50°C for 1 min, −72°C for 1 min for 35 cycles, 72°C for 10 min.

Dye primer sequencing

Sequencing products were obtained in both forward and reverse directions using Prism Big dye primer version 3.0 kits with −20 M13 and M13 reverse primers. Sequencing was performed according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Peak heights were determined from ABI trace files using Vector NTI 5.0. Values from forward and reverse strand sequencing were averaged and correlated with those obtained from control samples.

Clonal env sequencing

RT–PCR was performed on four plasma RNAs from four timepoints: 19 July 2001, 22 October 2001, 30 November 2001, and 7 February 2002. A nested protocol was used as described above using the V3 outer and inner primers. PCR products were cloned into TOPO cloning vectors according to the manufacturer's protocol, and 20 clones were analysed using dye terminator sequencing using the ABI prism 3100 genetic analyser.

Length polymorphism analysis of V4–V5 coding region of HIV-1 gp120

The env primers used in the PCR were: V3hln and V45-407. The primer V3hln was labelled with a fluorophore, 6-carboxy-fluorescein, at the 5’ end (PE Biosystems, Foster City, CA, USA). The PCR was a combined RT–PCR using Finzyme Robust RT–PCR (Finzymes, Espoo, Finland), and consisted of 48°C for 45 min, 94°C for 2 min, and then 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min. PCR-amplified fluorescently labelled products were separated on a 6% denaturing polyacrylamide gel using an automated sequencer (ABI PRISM 3100; PE Biosystems) and analysed using the GeneScan program (PE Biosystems).

Virosq sequencing


Primers used

RTF-1c: 5’–ttggaagaaattttgaactcag–3’

RTB-1c: 5’–gggtcaatactacgtggaacgg–3’

M13F-5RT: 5’–tggaaaaacgacgcctgtaaatactttc–3’

M13R-5RT: 5’–caggaaagcagccagttcagttttggc–3’

M13–20: 5’–gttaaaaagcagcggcag–3’

M13 reverse: 5’–caggaaagcagccagttcagttttggc–3’

V3 fout: 5’–caagatctccctgagcaaat–3’

V3 bout: 5’–attacagtagaaaaatctccct–3’

V3 fin: 5’–gaacaggagaggctgactcagatcaaat–3’

V3 bin: 5’–gcctaaagttctcggagctctctgag–3’

V45–407: 5’–attacactccatattgtgc–3’

V3hln: 5’–attacactctccatattgtgc–3’

Discussion

We present one case of HIV-1 superinfection by two HIV variants from the same viral clade in the absence of antiviral treatment. These data indicate that 4 months after infection by drug-resistant HIV this patient was infected by a second, drug-sensitive virus. This observation could alternatively be explained as co-infection with two divergent viruses, followed by late outgrowth of the variant, which was initially a minor population.

Discriminating co-infection from superinfection relies on the exclusion of a mixed viral population at the earliest timepoint post-infection. Because the detection threshold for both the dye primer sequencing and sequencing of 20 clones per timepoint is only 5–10% of the minor variant, we applied a more sensitive technique to detect population mixtures. Length polymorphism detection via Genescan technology is a highly sensitive tool for the detection of minority populations. The sensitivity for the detection of length polymorphisms goes down to one single nucleotide deletion or insertion within a PCR product [13]. Using this technology, we confirmed that the viral population in plasma at the three earliest timepoints were homogeneous. Therefore, with reasonable certainty, we excluded the possibility that this patient had been co-infected. A different viral population was dominant at timepoint 4 and thereafter. Genescan analysis in one experiment demonstrated the co-circulation of both viral populations at the earliest timepoint after the presumptive superinfection event. As expected from the results of the clonal sequencing, the shorter initial viral variant was present as a very small minor population based on relative peak heights. This provides further evidence that Genescan analysis permits the
detection of minority populations of less than 5%. The presence of the wild-type population as a minority species in the first 3 months is also less likely than superinfection, because the wild-type variant appears to be much more fit, with steady-state viral loads almost 100-fold greater than those of the initial drug-resistant variant. We did not find evidence for recombination in our case. Differences between the initial and superinfecting virus were consistent with all available analyses on pol, V3 and V4–V5. However, follow-up has been limited to 12 months.

One important issue is how superinfection impacts disease progression. Data from animal models indicate that there is a finite initial period of greater susceptibility to superinfection. Animals that seem to be protected from superinfection after primary infection had higher survival rates than animals that became superinfected [14,15].

Infection by viral variants with differing replication capacities and their variable susceptibility to the host immune response might be expected to have a significant impact on disease progression. Indeed, in the case described here, an abrupt increase in plasma viremia occurred coincident with the appearance of the second variant and consistent with the hypothesis that this second variant had greater in-vivo fitness than the initial, drug-resistant virus. The theoretical acceleration of disease progression that might result from a higher post-superinfection viral setpoint appears to be reflected in the steeper trajectory of the CD4 cell decline in this patient after superinfection.

A second issue of importance is the impact of superinfection on treatment response. An obvious scenario for concern is that of a patient with drug-sensitive virus responding well to therapy, who then becomes superinfected with drug-resistant virus. The transmission of drug-resistant virus is a common event [16,17]. However, the case described here highlights a more insidious danger arising as a consequence of superinfection. In this case, standard drug susceptibility testing at late timepoints would fail to detect the occult drug-resistant virus. Nevertheless, if this patient were to initiate therapy, it seems likely that drug-resistant virus would quickly re-emerge.

Our report coincides with three recent publications and one other report of apparent superinfection [9–11,18]. Two of the published accounts reported superinfection by virus from a different clade [9,10], whereas in this and another recently published report [11], superinfection appeared to be occurring with related virus from the same clade. In the case described by Altfeld et al. [11], the second infection occurred during a period of structured treatment interruption, despite strong immune control of the initial virus strain. This observation and the demonstration of intraclade superinfection indicates that the anti-HIV immune responses are narrowly directed, with worrying implications for vaccine design. Our case differs from most of the others and is similar to only one other case [18], in that it demonstrates intraclade superinfection that occurred in the absence of the modulation of natural disease by therapy or vaccination. Studies in larger cohorts as well as a more detailed analysis of humoral and cellular immune responses are needed to determine the true incidence of superinfection events and the underlying mechanisms. Together, these recent reports suggest that superinfection may occur more commonly than has previously been assumed, which has broad implications for HIV treatment, epidemiology, vaccine development and pathogenesis.

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Conflicts of interests statement: D.R. is a consultant for ViroLogic.

References


