Gag sequence variation in a human immunodeficiency virus type 1 transmission cluster influences viral replication fitness

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Three men from a proven homosexual human immunodeficiency virus type 1 (HIV-1) transmission cluster showed large variation in their clinical course of infection. To evaluate the effect of evolution of the same viral variant in these three patients, we analysed sequence variation in the capsid protein and determined the impact of the observed variation on viral replication fitness in vitro. Viral gag sequences from all three patients contained a mutation at position 242, T242N or T242S, which have been associated with lower virus replication in vitro. Interestingly, HIV-1 variants from patients with a progressive clinical course of infection developed compensatory mutations within the capsid that restored viral fitness, instead of reversion of the T242S mutation. In HIV-1 variants from patient 1, an HLA-B57+ elite controller, no compensatory mutations emerged during follow-up.

Previously, we reported on an HLA-B57+ long-term elite controller (patient 1) and his long-term HIV-negative partner (patient 2) who engaged in a triangular relationship with an HIV-positive partner (patient 3), leading to superinfection of patient 1 and HIV-1 infection of patient 2. After superinfection, patient 1 recovered with relative viraeic control, whereas patients 2 and 3 consistently had high viral loads and rapidly declining CD4+ T-cell counts and initiated combination antiretroviral therapy 2.4 and 3.7 years after seroconversion, respectively (Rachinger et al., 2008, 2011).

In the present study, we studied sequence variation in the portion of the gag gene encoding the HIV-1 capsid protein from these three patients and determined the impact of the observed variation on viral replication fitness. Written informed consent from all three patients was obtained. The study was approved by the Academic Medical Center institutional medical ethics committee.

Longitudinal gag sequences were previously generated (Rachinger et al., 2011) both from viral RNA in plasma and from clonal HIV-1 variants isolated from PBMCs (GenBank accession numbers JF278423–JF278560). The follow-up period was 25 months after superinfection for patient 1, and respectively 25 and 38 months after seroconversion for patients 2 and 3. HLA genotypes are as follows: patient 1: HLA-A0101, HLA-A0301, HLA-B5701 and HLA-B3501; patient 2: HLA-A0101, HLA-A0201, HLA-B0801 and HLA-B4402; patient 3: HLA-A0201, HLA-A2902, HLA-B4402 and HLA-B4403.

During human immunodeficiency virus type 1 (HIV-1) infection, new viral variants emerge continuously, due to the error-prone nature of the HIV-1 reverse transcriptase and high viral turnover. The development of a highly diverse quasispecies supports adaptation to the host environment and ongoing selection of the fittest virus variants. Cytotoxic T-lymphocyte (CTL) responses play a key role in controlling virus replication during the asymptomatic phase of HIV-1 infection, and evasion of CTL surveillance has a strong influence on virus evolution. However, amino acid changes associated with CTL escape may come at a fitness cost, particularly when the mutation is situated in a conserved region such as gag. Indeed, certain CTL-escape mutations in epitopes restricted by protective HLA alleles, of which the T242N escape mutation within the gag TW10 epitope restricted by HLA-B57 is the most well-studied, have been shown to reduce virus replication capacity (Martinez-Picado et al., 2006; Navis et al., 2007). The fitness cost of the T242N CTL-escape mutation can be overcome by compensatory mutations in gag (Brockman et al., 2007). Furthermore, it has been reported that the T242N mutation reverts rapidly upon transmission to an HLA-B57+ individual (Friedrich et al., 2004; Leslie et al., 2004).

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A supplementary table is available with the online version of this paper.
We here analysed the gag sequences for patient-specific sequence variation within the capsid (aa 133–339 of Gag). The capsid sequences of all three patients were very similar, as the patients were infected with the same viral variant (Table S1, available in JGV Online).

Comparing the sequences between the three patients, we observed patient-specific sequence variation at three positions (Table 1, Fig. 1a). Viral sequences from patient 1 contained the T242N CTL-escape mutation within the HLA-B57-restricted TW10 epitope at all time points studied and no additional sequence variation was observed. All viral sequences obtained from patients 2 and 3 contained the T242S mutation. An additional H219Q mutation emerged in sequences from patients 2 and 3, respectively 19 and 13 months after seroconversion. A third mutation (S310T) was observed in viral sequences obtained from patient 3, 19 months after seroconversion (Table 1, Fig. 1a). During follow-up of patient 2, the frequency of viral variants carrying the H219Q mutation increased over time and, 25 months after seroconversion, 50% of the viral variants contained this mutation (Fig. 1a). Viral variants isolated from patient 3 showed the independent emergence of viral variants carrying either the H219Q or S310T mutation 13 months after seroconversion and these variants coexisted up to 23 months after seroconversion. The H219Q variant became the dominant viral variant after 34 months of follow-up, whereas viral variants containing the S310T mutation were no longer observed. Viral variants carrying both the H219Q and S310T mutation emerged 23 months after seroconversion and remained present during follow-up (Fig. 1a). The emergence of the H219Q and/or S310T mutations in viral variants of both patients 2 and 3 was associated with a progressive disease, as demonstrated by declining CD4+ T-cell counts (Fig. 1b) and increasing viral load (Fig. 1c).

To evaluate the effect of the observed sequence variation in capsid on viral replication kinetics, NL4-3.Ba-L molecular clones containing patient-specific mutations were constructed. The gag region obtained from patient 1 was cloned into the pGEM-T Easy vector system (Invitrogen) and mutations were introduced using site-directed mutagenesis (QuickChange kit; Stratagene). The gag region of molecular clone NL4-3.Ba-L was removed using restriction enzymes BssHII and Apal (Invitrogen). Replicating virus variants were produced by co-transfection of NL4-3.Ba-L Δgag and the constructed gag regions containing patient-specific mutations into 293T cells. Virus titres of the produced mutant viruses were determined on PBMCs as TCID₅₀ values (van’t Wout et al., 2008).

The in vitro replication capacity of the constructed viruses was determined on phytohaemagglutinin (PHA)-stimulated pooled PBMCs from 10 healthy donors. In brief, 2 × 10⁶ PHA-stimulated PBMCs were inoculated with 50 TCID₅₀ virus for 2 h. Afterwards, the cells were washed, resuspended in 1.5 ml culture medium containing 20 U interleukin-2 ml⁻¹ and divided between three wells of a
24-well plate at a cell density of $6.7 \times 10^5$ in 500 μl each. Cultures were continued for 18 days and $5 \times 10^5$ PHA-stimulated PBMCs from the same PBMC pool were added on days 5, 8, 11 and 14 to propagate the culture. Virus replication was analysed in daily culture supernatant samples (35 μl) by an in-house p24 ELISA (Tersmette et al., 1989). On day 18, total DNA was isolated from the cultures; gag was sequenced as described previously (Navis et al., 2007) to confirm the introduction of mutations.

The replication capacity of the constructed viruses differed substantially, with a relatively low replication capacity of the virus carrying 242N found in viral sequences obtained from patient 1, and increasing replication capacity for capsid variants containing specific mutations from patients 2 and 3 (Fig. 2a). Comparing the mean p24 levels on day 14, we observed that viral variants carrying 242N or 242S had significantly lower p24 production than viral variants that additionally had the H219Q and/or S310T mutations.
mutations. The highest mean p24 value was observed for viral variants carrying 242S, H219Q and S310T (Fig. 2a).

To investigate further the effect of the observed mutations on replication kinetics, NL4-3.Ba-L molecular clones carrying 242N, 242S or 242T in combination with amino acid variation at positions 219 (H and Q) and/or 310 (S and T) were constructed and replication kinetics were determined as described above. The H219Q mutation increased p24 production of viral variants carrying either 242N ($P=0.0015$) or 242S ($P=0.0410$), restoring virus replication to levels comparable to those of the virus variant containing the HIV-1 subtype B consensus sequence 242T. The H219Q mutation had no significant effect on the replication kinetics of the 242T variant (Fig. 2b).

The S310T mutation alone had no effect on the replication of the 242N virus variant. A trend towards higher p24 levels was observed after introduction of the S310T mutation into the 242S variant ($P=0.1058$); however, this mutation significantly reduced replication of the 242T variant ($P=0.0352$; Fig. 2b).

When the S310T mutation was introduced into the 242S variant in combination with the H219Q mutation, significantly higher virus replication was observed compared with the viral variants containing 242S alone ($P=0.0219$) or 242S in combination with S310T ($P=0.0296$). However, the mean p24 level did not differ significantly from the mean p24 level of variants carrying 242S in combination with H219Q alone. Whilst virus replication of the 242N variant increased significantly after introduction of the H219Q mutation, the S310T mutation alone or in combination with the H219Q mutation did not affect virus replication. Addition of both the H219Q and S310T mutations to the 242T variant showed a trend towards lower p24 levels ($P=0.0742$; Fig. 2b).

Here, we studied patient-specific sequence variation within the capsid region of viral variants isolated from three men who engaged in a triangular relationship with proven HIV-1 transmission. Despite being infected with the same HIV-1 variant, these individuals showed marked differences in their clinical course of infection. Patient 1 carries the protective HLA-B57 allele, and the T242N escape mutation in the TW10 epitope was found in all viral sequences. This CTL-escape mutation is associated with impaired virus replication (Martinez-Picado et al., 2006; Navis et al., 2007) and several mutations have been described to compensate for the fitness cost (Brockman et al., 2007; Navis et al., 2007). Interestingly, we did not observe the emergence of compensatory mutations in viral variants obtained from patient 1, possibly explaining the relatively low replication capacity of the virus in vitro and the low viral load in vivo.

The absence of compensatory mutations cannot be explained by CTL pressure, as the potential compensatory mutations do not influence epitope processing or binding to HLA class I molecules of patient 1. It remains unclear why compensatory mutations did not emerge. However, strong control of virus replication, as demonstrated by low viral loads in vivo, could delay the development of mutations, suggesting that compensatory mutations may develop during longer follow-up.

The capsid sequences obtained from patients 2 and 3 all have the 242S mutation. This mutation has been described to be a transient intermediate when T242N reverts upon transmission to an HLA-B57- recipient, although reversion to 242T is observed more frequently (Crawford et al., 2007; Novitsky et al., 2010). Although the donor of HIV-1 infection of patient 3 is unknown, the presence of the T242S mutation may indicate transmission from an HLA-B57+ donor to patient 3, and subsequent transmission of the 242S mutation to patients 1 and 2. In all gag sequences from the three patients, mutations I147L and I223V are present. These mutations have been suggested to have a

![Fig. 2. Replication kinetics of mutant capsid virus variants in vitro.](http://vir.sgmjournals.org)
compensatory effect on the fitness cost of the T242N mutation (Brockman et al., 2007; Durand et al., 2010), which may also suggest an HLA-B57+ source of infection for patient 3. However, other compensatory mutations, such as G248A, H219Q and M228I (Brockman et al., 2007; Navis et al., 2007), are absent.

Our data indicate that the 242S mutation comes at a fitness cost, resulting in a low replication capacity comparable to that of virus variants carrying 242N. Surprisingly, we did not observe the S242T reversion in any of the viral sequences obtained from patients 2 and 3, raising the question of why reversion to 242T has not occurred. Additional sequence variation was observed at positions 219 and 310, which increased the replication kinetics of the 242S variant to levels comparable to those in the wild-type 242T virus; reversion may therefore no longer result in a substantial increase in viral fitness. Moreover, introduction of the H219Q and S310T mutations into the 242T variant seems to decrease the replication kinetics, indicating that these mutations may hamper the S242T transition. A similar observation was made by Novitsky et al. (2010), who described fixation of 242S in the quasispecies of two out of seven HLA-B57/B5801+ individuals infected with a 242N subtype C variant after compensation of the fitness cost associated with the 242S mutation by N147L and N252G.

It has been shown that viral variants containing the N242T reversion emerge and become dominant within the first year after transmission (Crawford et al., 2007; Leslie et al., 2004; Novitsky et al., 2010). In our patients, the compensatory mutations H219Q and S310T emerged approximately 1.0–1.5 years after infection, and it is unclear why transition of 242S to 242T was not observed before the compensatory mutations occurred, especially in the absence of the HLA-B57 allele. Neither 242S nor 242T influences epitope processing or binding to HLA molecules expressed by patients 2 and 3, and CTL pressure cannot explain fixation of 242S and the absence of 242T in the viral quasispecies of these patients.

Viral variants carrying 242S coexisted with variants carrying one or both of the compensatory mutations in patients 2 and 3. However, during prolonged follow-up of patient 3, the 242S variant without additional compensatory mutations became a minor variant and was no longer detected 38 months after seroconversion, which is probably due to selection for the fitter viral variants carrying 242S and H219Q or 242S, H219Q and S310T. Loss of the 242S variant was not observed in patient 2, which may be due to limited follow-up. Furthermore, the viral variant containing the S310T mutation alone was no longer observed in viral sequences obtained from patient 3 at the end of follow-up. Indeed, we observed that the S310T mutation does not significantly increase replication fitness of the 242S and the 242S–H219Q viral variant, which may indicate that the S310T mutation does not have a selective advantage with regard to viral fitness.

In patient 3, and to a lesser extent in patient 2, an increase in viral load was observed prior to the emergence of viral variants containing the H219Q and/or S310T mutations. The increase in viral load may have accelerated the development of compensatory mutations and, subsequently, the emergence of viral variants with an increased replication capacity. In turn, the emergence of viral variants with higher replication fitness may have contributed to disease progression.

In conclusion, we here studied three individuals who engaged in a triangular relationship and showed large variation in the clinical course of HIV–1 infection. We analysed the impact of mutations in the capsid region on viral replication capacity and demonstrated that viruses from all three patients contained a mutation at position 242 (242N or 242S) in the capsid protein, impairing viral replication capacity. The viral fitness was restored by compensatory mutations in patients 2 and 3, who experienced a progressive course of infection, whereas in viral variants from patient 1, an HLA-B57+ elite controller, no compensatory mutations emerged during follow-up.

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**References**


