The evolution of human immunodeficiency virus type-1 (HIV-1) envelope molecular properties and coreceptor use at all stages of infection in an HIV-1 donor–recipient pair

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ABSTRACT

To trace the evolutionary patterns underlying evolution of coreceptor use within a host, we studied an HIV-1 transmission pair involving a donor who exclusively harbored CCR5-using (R5) variants throughout his entire disease course and a recipient who developed CXCR4-using variants. Over time, R5 variants in the donor optimized coreceptor use, which was associated with an increased number of potential N-linked glycosylation sites (PNGS) and elevated V3 charge in the viral envelope. Interestingly, R5 variants that were transmitted to the recipient preserved the viral characteristics of this late stage genotype and phenotype. Following a selective sweep, CXCR4-using variants subsequently emerged in the recipient coinciding with a further increase in the number of PNGS and V3 charge in the envelope of R5 viruses.

Although described in a single transmission pair, the transmission and subsequent persistence of R5 variants with late stage characteristics demonstrate the potential for coreceptor use adaptation at the population level.

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Introduction

Human Immunodeficiency Virus type 1 (HIV-1) target cell entry is mediated by the interaction of the viral envelope protein with CD4 and a coreceptor on the target cell surface (Alkhatib et al., 1996; Berson et al., 1996; Dalgleish et al., 1984; Klatzmann et al., 1984). The main coreceptors used by HIV-1 in vivo are the chemokine receptors CCR5 and CXCR4 (De Roda Husman et al., 1999; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996).

CCR5-using (R5) viruses predominate in the early stages of HIV-1 infection irrespective of the route of transmission (Casper et al., 2002; Husson et al., 1995; Keele et al., 2008; Salazar-Gonzalez et al., 2008; Scarlatti et al., 1993; Van’t Wout et al., 1994) and persist throughout the course of the disease (Connor et al., 1997; Scarlatti et al., 1997; Schuitemaker et al., 1992). The biological properties of R5 viruses commonly evolve throughout the natural course of infection (Jansson et al., 1996, 1999; Koning et al., 2003; Kwa et al., 2003; Repits et al., 2005). Indeed, late stage R5 HIV-1 variants show more rapid replication, higher cytopathicity and a more efficient use of CCR5; the latter is reflected by a decreased sensitivity to inhibition by CCR5 antagonists and the ability to use CCR5–CXCR4 chimeric coreceptors (Jansson et al., 1999; Koning et al., 2003; Kwa et al., 2003; Repits et al., 2005). In the absence of antiretroviral therapy, about 50% of individuals progress to AIDS in the sole presence of R5 HIV-1 variants (De Roda Husman et al., 1999; Jansson et al., 1999; Karlsson et al., 1994a; Koot et al., 1993).

The other 50% of HIV-1 subtype B infected individuals progress to AIDS in the presence of CXCR4-using variants. The emergence of CXCR4-using HIV-1 generally precedes a faster CD4+ T cell decline and accelerated disease progression (Connor et al., 1997; Karlsson et al., 1994b; Koot et al., 1993, 1996; Schuitemaker et al., 1992). Despite years of extensive research, the determinants for the emergence of CXCR4-using variants remain largely unresolved.

The evolution of R5 variants throughout infection and the rare presence of CXCR4-using variants at a very early stage have raised the question whether viral characteristics of early stage viruses are...
advantageous for transmission. However, it is currently unknown whether the phenotype of late stage R5 variants can be preserved upon transmission to a new individual, and up to what level it contributes to shape the viral population in the new host (i.e. efficiency of coreceptor use, emergence of CXCR4-using variants, evolutionary rate or development of resistance to a neutralizing antibody response).

Here, we studied HIV-1 evolution in a donor-recipient pair in which the donor harbored only R5 variants during his entire course of infection, whereas in the recipient CXCR4-using variants eventually emerged. We compared evolutionary rates and dN/dS ratios based on gp120 envelope sequences, efficiency of CCR5 use, and envelope molecular characteristics of longitudinally obtained R5 variants, and in particular from R5 variants obtained prior and after the transmission event, from both donor and recipient. With the longitudinal analysis of changes in viral phenotype and envelope molecular characteristics in relation to HIV-1 evolutionary history in this transmission pair, we attempt to define the role of viral factors during transmission and in the evolution of the HIV-1 population within a host.

Results

Phylogenetic analysis of env sequences demonstrates HIV-1 transmission from an HIV-1 infected individual with R5 variants only, to an individual in whom eventually CXCR4-using HIV-1 variants developed

A Maximum likelihood (ML) phylogenetic tree based on env gp120 C2–C4 sequences from 103 participants of the ACS merged with a highly related but not epidemiologically linked international reference panel of 148 sequences, revealed well-supported clustering of subject 19858 and subject 19308 derived sequences (data not shown). Moreover, sequences from subject 19308 were nested in a larger cluster of sequences that were exclusively derived from subject 19858 (both clusters supported by bootstrap analysis), suggesting that direct HIV-1 transmission had occurred from subject 19858 (donor) to subject 19308 (recipient). Interestingly, routine testing using the MT-2 assay for the presence of CXCR4-using variants in the donor never yielded a positive test, suggestive of the absence of CXCR4-using variants, while the recipient tested positive in the MT-2 test from 3.4 years after seroconversion onwards.

To explore in more detail the HIV-1 evolutionary history in these two subjects we performed ML as well as Bayesian MCMC analysis of the two subject’s viral env sequences. The ML tree (Supplementary Fig. A1A) and the Bayesian maximum clade credibility (MCC) tree [Fig. 2A] topologies for the alignment of env gp120 C1–V5 sequences derived from clonal HIV-1 variants supported a single transmission event from donor to recipient, showing a temporal structure of sequences and monophyletic clustering of the recipient sequences, which branched off from late donor sequences. Moreover, the phylogenies also demonstrated monophyletic clustering of the recipient’s CXCR4-using variant sequences, which descended from his R5 variants. The ML tree (Supplementary Fig. A1B) and Bayesian MCC tree (Fig. 2B) topologies for the shorter alignment of env gp120 C2–C4 sequences derived from clonal HIV-1 variants and viral RNA in serum also showed a temporal structure of sequences. However in these trees, recipient sequences did not cluster monophyletically, as single donor sequences (three in ML tree, five in MCMC tree) from later time points fell within the recipient’s cluster. Additionally, the majority of sequences with predicted and/or in vitro tested CXCR4 use of the recipient were part of a main CXCR4-using sequence cluster but three of them constituted a separate cluster. Interestingly, these three sequences were derived from serum at 10 or 2 months before the estimated date of emergence of CXCR4-using variants and provided discordant results by the V3 coreceptor prediction methods. This may imply that these viral variants from serum were intermediates between CCR5- and CXCR4-using viruses that failed to produce replication-competent progeny. This was further supported by the absence of viral descendants from these three virus variants. In conclusion, analysis of env gp120 C1–V5 sequences derived from clonal HIV-1 variants, the most informative alignment, clearly supports a single transmission event from donor to recipient. Due to lower evolutionary information, the shorter alignment of env gp120 C2–C4 sequences derived from clonal HIV-1 variants and viral RNA in serum did not yield accurate reconstructions of the phylogenetic relationships. However, the clustering of donor sequences from late time points with recipient sequences from early time points further underscores the high sequence similarity between viral populations isolated from donor and recipient, and hence their close genetic relationship. Differences between sequences obtained from RNA in serum and from isolated clonal HIV-1 variants from PBMC could be attributed to the amount of sequence information rather than to the source from which sequences were obtained. Therefore, env gp120 C1–V5 sequences from clonal HIV-1 variants were used for subsequent analysis as this allowed the study of biological properties of the virus in the context of the original genetic background.

Bayesian MCMC analysis based on env gp120 C1–V5 sequences from clonal HIV-1 variants estimated time to the most recent common ancestor (TMRCA) of the recipient at 98 (95% credible intervals (CI) = 93.9–99.8) months after SC of the donor. This estimate dates the TMRCA for the recipient, which puts a lower bound on date of HIV-1 transmission, back to around 12 July 1991, which corresponds to the SC date of the recipient (14 July 1991). Despite the absence of epidemiological data confirming the relationship between those two individuals, this strongly supports the transmission of HIV-1 between the two individuals, as was concluded from the phylogenetic analysis.

Evolutionary rate of HIV-1 variants from donor and recipient

The mean evolutionary rate in the HIV-1 transmission chain was estimated to be 0.00012 (95% CI = 0.0009–0.0015) substitutions per
site per month for the env gp120 C1–V5 sequences from clonal HIV-1 variants, but the coefficient of variation (0.44 (95% CI = 0.22–0.71)) indicated considerable rate variation among branches, which justifies the use of a relaxed clock for the transmission history. To assess whether evolutionary rates were different between HIV-1 variants in the donor and in the recipient, we performed Bayesian MCMC analysis of the clonal HIV-1 variant sequences for each subject separately. For the recipient, we also performed an additional analysis solely on the clonal R5 variant sequences. Bayes factor testing indicated that a strict clock provided a good fit for the donor, but a relaxed clock appeared to be more appropriate for the recipient (for all clonal HIV-1 variant sequences as well as clonal R5 variant sequences separately, data not shown). For both the relaxed and strict clock estimates (Fig. 3), the mean evolutionary rate of the donor’s R5 variant sequences was 1.6-fold lower than the estimated evolutionary rate of the recipient’s R5 and CXCR4-using variant sequences. However, the differences were less pronounced when the mean evolutionary rate of the donor’s R5 variant sequences was compared to the mean evolutionary rate of the recipient’s R5 variant sequences (1.4 and 1.3-fold difference for relaxed and strict clock estimates, respectively) and credible intervals were even more broadly overlapping.

**Estimation of dN/dS ratios of HIV-1 variants from donor and recipient**

To estimate dN/dS ratios, three different models were fitted to the alignment of donor and recipient env gp120 C1–V5 sequences derived from clonal HIV-1 variants: Model A (a single dN/dS ratio for all branches), Model B (separate dN/dS ratios for branches in the donor and recipient clade) and Model C (separate dN/dS ratios for branches in the donor clade, in the recipient R5 variant clade and in the recipient CXCR4-using variant clade) (Table 2). Likelihood ratio testing revealed that Model B provided a significantly better fit to the data than the simpler single ratio model (Model B, \( p = 0.012 \), and that Model C did not provide any improvement in fit respect to Model 2, \( p = 0.391 \)). Although dN/dS ratios for both subjects were < 1 under Model B, indicating a predominantly negatively selection, the higher dN/dS ratio in the recipient compared to the donor may be attributed to a more neutral evolution in general or a bout of positive selection in the viral evolutionary history in this subject. Given that Model 3 did not lead to a better fit, we cannot conclude any difference in dN/dS between R5 and CXCR4-using sequence clades and that selection pressure on the CXCR4-using viral population, although
Fig. 2 (continued).
contributing to the overall dN/dS ratio for the recipient, is not solely responsible for the differences with the dN/dS ratio of the donor.

To examine in more detail the differences observed in dN/dS ratio estimates between donor and recipient, we estimated the branch lengths of the MCC tree based on expected synonymous (E[S]) and nonsynonymous (E[N]) changes. For this we used a codon model that allows for a different synonymous and nonsynonymous substitution rate along each branch (a `local` codon model, (Kosakovsky Pond et al., 2009)) (Supplementary Figs. A2 A and B). We subsequently plotted E[S] and E[N] divergence over time in Fig. 4. In general, E[S] and E[N] variation occurred roughly at similar pace, as reflected in the parallel increase in E[S] and E[N] during the entire follow-up of the donor, and during the follow-up after the second time point of the recipient. However, a marked increase in E[N] could be observed between the first and second sampling time point of the recipient (101 and 126.6 months since SC of the donor, respectively) and, in fact, between these points E[N] divergence exceeded E[S] divergence. This is a clear signal for a selective sweep between these time points, in other words, it indicates that during that time frame viral envelopes of the recipient were subject to positive selection. The E[N] and E[S] trees also show that while there is a long branch between these time points in the E[N] tree, there is hardly any divergence in the E[S] tree. This suggests that the increase in E[N] between the first two time points of the recipient is responsible for the higher dN/dS in the donor as compared to the recipient. The fact that this positive selection is restricted to the time between these time points explains why the evolutionary rate is only moderately faster in general between donor and recipient and why dN/dS is not particularly higher for the CXCR4-using variants.

**CCR5 use of R5 variants from donor and recipient**

In order to determine the efficiency of CCR5 use of R5 variants, before and after transmission, we tested the sensitivity of longitudinally obtained R5 variants from donor and recipient to inhibition by two anti-CCR5 monoclonal antibodies directed against the CCR5 N-terminal domain (MAb RoAb13) and the second extracellular loop (ECL2) of CCR5 (MAb RoAb952). A minimum of two and a maximum of six R5 variants per time point were tested (Table 1).

R5 variants from the donor showed an increasing resistance to MAb RoAb13-mediated inhibition over time (Fig. 5A), as shown by a significant increase in IC50 values per time point (P=0.0012), but not to MAb RoAb952-mediated inhibition (Fig. 5B). However, we did observe a trend towards higher IC50 values of MAb RoAb952 for R5 variants from the later time point (111 months after SC; average IC50 (ng/ml)= 850.8±724.8) as compared to R5 variants isolated at earlier time points (40, 66 and 90 months after SC; average IC50 (ng/ml) = 138±102.3; P=0.0685).

A decrease in IC50 values between R5 variants isolated at the first time point and at later time points from the recipient was observed for the two anti CCR5 MAbs (Figs. 5A and B), although these differences did not approach statistical significance.

R5 variants from donor and recipient isolated close to the transmission event showed a similar sensitivity to inhibition by the two MAbs (Figs. 5A and B), as there were no significant differences between IC50s of the earliest R5 variants from the recipient and IC50s of R5 variants isolated from the donor prior (90 months after SC) and after (111 months after SC) the...
transmission event (98 months after SC). This leads us to conclude that these variants used CCR5 with the same efficiency.

Envelop molecular properties of HIV-1 variants from donor and recipient

Variation in charge and PSSM score of the env gp120 V3 region and in the number of Potential N-linked Glycosylation Sites (PNGS) of the env gp120 C1–V5 region were studied, over the course of infection, for R5 variants from the donor and for R5 and CXCR4-using variants from the recipient.

The charge of the V3 regions for R5 variants from both donor and recipient increased over time (P = 0.03 and P = 0.032 respectively; Fig. 6A) but was always lower than for CXCR4-using variants of the recipient. Whereas no change in PSSM score was observed for R5 variants from the donor, PSSM score for the R5 variants from the recipient significantly increased over time (P < 0.0001; Fig. 6B). R5 variants isolated at the last three time points from the recipient had overall more positively charged V3 regions and higher PSSM score than R5 variants from the donor (P = 0.0001 in both cases).

An overall increase in number of PNGS over time was observed in R5 variants of both patients (P = 0.031 and P = 0.002 respectively; Fig. 6C). For R5 variants of the recipient, this increase was more pronounced, reaching the maximum peak at the second time point, after which a decrease in number of PNGS was observed in both R5 and CXCR4-using variants.

The V3 charge for the earliest R5 variants from the recipient was similar to the V3 charge for the R5 variants isolated from the donor prior (90 months after SC) and after (111 months after SC) the transmission event. The PSSM score between the earliest R5 variants from the recipient and the R5 variants from the donor isolated prior (90 months after SC) transmission was similar, but the PSSM score of the R5 variants isolated from the donor after (111 months after SC) transmission was significantly higher than for the earliest R5 variants of the recipient. Moreover, the number of PNGS was also similar for the earliest R5 variants from the recipient and the genetically most related R5 variants from the donor, which according to the phylogenetic analysis (Fig. 2A and Supplementary Fig. A1A), were the variants isolated after the transmission (111 months after SC). However, the number of PNGS of the earliest R5 variants from the recipient was lower than in R5 variants isolated from the donor prior the transmission event (90 months after SC).

Discussion

An HIV-1 transmission pair provided the unique opportunity to study in detail the evolution of highly related HIV-1 variants in two unrelated host environments after transmission from one individual, who only harbored R5 variants during his entire disease course, to another individual in whom CXCR4-using variants emerged after a 3.4 year-period during which only R5 variants were detected. Phylogenetic analysis supported a single transmission of R5 variants from donor to recipient and revealed that CXCR4-using variants that emerged in the recipient descended from the transmitted and initially expanded R5 viral population. This data implies that CXCR4-using variants can indeed emerge “de novo” and are not necessarily acquired by transmission.

An increase in efficiency of HIV-1 CCR5 use was observed over time in the donor. Interestingly, the earliest recipient’s R5 variants displayed a similar efficiency of CCR5 use as these late R5 variants from the donor, which were isolated close to the moment of transmission, and maintained this late-stage phenotype during follow-up. At similar time points in the course of infection, CD4+ T cell counts and viral load (VL) were respectively lower and higher in the recipient as compared to the donor (Fig. 1). The lower CD4+ T cell counts, and thus the lower amount of target cells in the recipient may have facilitated the persistence of R5 variants with highly efficient CCR5 use after transmission, by itself giving rise to high VL. Alternatively or in addition, the lower CD4+ T cell counts and, consequently, the lower target cell availability already from the beginning of the infection, could at least partially explain the initially stronger selection pressure on HIV-1 in the recipient. Indeed, although selection pressure on the HIV-1 envelope was roughly similar in donor and recipient, a stronger positive selection pressure was observed on viruses of the recipient during the 2.4 years immediately after transmission, which was reflected in an overall higher ratio of nonsynonymous versus synonymous substitution rates (dn/ds). This explains why the viral evolutionary rate, which is determined by the viral mutation rate, the viral generation time, and the selective pressure acting on the virus, is only moderately faster in the recipient than in the donor.
Given the high mutation rate of HIV-1, and considering that few amino acid changes in the envelope V3 region are sufficient for a switch from CCR5 to CXCR4 coreceptor use (Chesebro et al., 1996; Cordonnier et al., 1989; De Jong et al., 1992; Harrowe and Cheng-Mayer, 1995; Mosier et al., 1999; Shimizu et al., 1999; Shioda et al., 1992), emergence of CXCR4-using variants—if beneficial for the virus—would be expected to occur rapidly and relatively early in the course of infection in every patient. However, absence of CXCR4-using variants during the entire disease course is rather common (Koot et al., 1993, 1999), and when CXCR4-using variants do emerge, this generally occurs at a late stage of disease. The lower replication rate, reduced efficiency of coreceptor use and higher susceptibility to neutralizing antibodies (Kuiken et al., 1999; Shimizu et al., 1999; Shioda et al., 1992), emergence of CXCR4-using variants in the recipient coincides with a decline in CrNA (Euler et al., 2010) and an increased susceptibility of coreceptor use (Chesebro et al., 1996; Cordonnier et al., 1999; Pastore et al., 2004; Van’t Wout et al., 1998) of variants that are in transition from CCR5 to CXCR4 use suggest that intermediate variants traverse a stage of reduced viral fitness. Therefore, additional compensatory mutations, which most likely are context dependent, may be required to create the background in which the essential V3 region mutations result in a successfully replicating CXCR4-using HIV-1 variant. In our study, the transmitted R5 variants, as a result of adaptation to the donor’s environment, may have had already a backbone with part of the mutations required for the transition to a CXCR4-using phenotype. Indeed, the gp120 envelope V3 regions of late R5 variants from the recipient were overall more positively charged than of R5 variants from the donor and had higher PSSM scores. The fact that the V3 region of a CXCR4-using envelope is generally more positively charged (De Jong et al., 1992; Fouchier et al., 1992, 1995; Shioda et al., 1992) and has a higher PSSM score (Jensen et al., 2003) may indicate that the envelope composition of the recipient’s R5 viruses may have been more similar to the one of a CXCR4-using virus. The complete absence of CXCR4-using variants in the donor despite sufficient follow-up time (5 years after the transmission event and 4 years after the last time point studied in the donor), implies that host factors are likely to play a role in the acquisition of CXCR4 use. Indeed, host factors such as coreceptor and IL-7 expression levels have previously been recognized as determinants in the evolution towards CXCR4 use (Brieu et al., 2011; van Rij et al., 2003). Moreover, the higher incidence of CXCR4-using HIV-1 variants when CD4⁺ T cell counts drop below 500 cells/μl (Koot et al., 1999) suggests that host immune surveillance may counteract the development of these viruses. The higher CD4⁺ T cell counts in the donor may therefore correlate with the absence of CXCR4-using variants in this patient. The actual host factors involved in the differential viral evolution in the partners of this particular transmission pair remain to be established.

The initial increase in PNGS in the viral envelope sequences was more pronounced in the R5 variants from the recipient than from the donor, and may reflect the continuous viral escape from the autologous humoral immune response in the host (Bunnik et al., 2008; Gao et al., 1997; Cheng-Mayer et al., 1999; Sagar et al., 2006; Saunders et al., 2005; van Gils et al., 2010, 2011; Wei et al., 2003). In line, serum from the recipient, but not the donor, had cross-reactive neutralizing activity (CrNA) (Euler et al., 2010 and submitted for publication). The emergence of CXCR4-using variants in the recipient coincides with a decline in CrNA (Euler et al., 2010) and a decrease in PNGS in both R5 and CXCR4-using variants. The decline in humoral immunity could be a sign of the impairment of the immune system, which, as discussed before, might be one of the factors favoring the emergence of CXCR4-using variants.

We show that recently transmitted R5 HIV-1 variants can have similar envelope molecular properties and efficiency of CCR5 use as late stage R5 variants. In agreement, VL set-point has recently been shown to be in
large part determined by viral genotype, and the heritability of this trait has been shown at the population level (Alizon et al, 2010). This implies that late stage adaptations that support virus replication, efficient target cell infection and evasion from the host immune response can be preserved upon transmission, which may contribute to the evolution of HIV-1 phenotype and the rise in VL set-point throughout the HIV-1 epidemic (Bunnik et al, 2010; Gras et al, 2009; Kawashima et al, 2009; van Manen et al, 2011). However, the increased risk of transmission in the acute phase relative to the chronic phase of infection, which plays a pivotal role in HIV-1 spread (Jacquez et al, 1994; Koopman et al, 1997; Leynaert et al, 1998; Pao et al, 2005; Pilcher et al, 2004; Yerly et al, 2001), could explain why the adaptation of HIV-1 at the population level seems to be a slow process.

Although focused on a single transmission case, we have shown here that a late stage viral phenotype and envelope molecular properties can be transmitted and preserved in the recipient. In-depth studies on HIV-1 transmission chains may advance our understanding of the evolutionary dynamics of HIV-1 molecular and biological properties within a host and at the population level throughout the epidemic history.

Material and methods

Study subjects and samples

Longitudinal cryopreserved peripheral blood mononuclear cells (PBMC) samples from two homosexual men, subject 19858 (donor) and subject 19308 (recipient), who participated in the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS, http://www.amsterdamcohortstudies.org), with an imputed or documented seroconversion (SC) date (Geskus, 2000), respectively, were used for this study. The donor remained therapy-naive while the recipient received changing antiretroviral therapy (AZT+3TC; ddI+d4T+Indinavir) starting at the third-before-last time point analyzed (50 months after SC). Time points studied are shown in Table 1. Longitudinal data on CD4+ T cell counts and plasma viral load are shown in Fig. 1.

The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set out in the declaration of Helsinki and were approved by the Academic Medical Center institutional medical ethics committee. Written informed consent was obtained from all cohort participants.

Isolation of replication-competent clonal HIV-1 variants from PBMC

Replication-competent clonal HIV-1 variants were isolated by cocultivation of serial dilutions of cryopreserved patient PBMC with PHA-stimulated PBMC obtained from HIV-1 seronegative donors as described previously (Schuitemaker et al, 1992; Van ’t Wout et al, 2008). From each time point, a median of 6 clonal HIV-1 variants (range, 4–15) was subjected to DNA isolation, PCR and sequencing (Table 1).

DNA isolation, PCR and env gp120 C1–V5 sequencing from replication-competent clonal HIV-1 variants from PBMC

Total DNA was isolated from 0.5–1×10⁸ HIV-1 infected cells using a modification of the L6 isolation method (Kootstra and Schuitemaker, 1998). Gp120 env PCR amplification was performed with one outer PCR with primers TB3 forward (5′-GGCTATTAGGACAATGCTAGT-TAGCC-3′) and OFM19 reverse (5′-GCACTAAAGGGAATGTTATT-TAGGCTTA-3′) and a nested PCR with primers env1aTOPO forward (5′-CACCCTGATGATCGTCATTGAAAGA-3′) and envN reverse (5′-CTGACCAAGGAGAAATGGGCTG-3′) using the Expand High Fidelity Taq Polymerase kit (Roche) and the following amplification cycles: 2 min 30 s at 94 °C, 9 cycles of 15 s 94 °C, 45 s 50 °C, 6 min 68 °C, 30 cycles of 15 s 94 °C, 45 s 53 °C, 6 min 68 °C, followed by 10 min at 68 °C and cooling to 4 °C. PCR products were purified using ExoSAP-IT (USB) according to the manufacturer’s protocol. Sequencing conditions were 5 min at 94 °C, 30 cycles of 15 s at 94 °C, 10 s at 50 °C, 2 min at 60 °C and 10 min at 60 °C. Sequencing of gp120 C1–V5 region corresponding to HXB2 nucleotide positions 6465 to 7636 was performed using BigDye Terminator v1.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems) according to the manufacturer’s protocol using the primers Seq1 forward (5′-TACATAATGTTGGGCCACCATGCC-3′), Seq4 reverse (5′-CTTGTATGTGGCCTGTGAC-3′). Seq5 forward (5′-GTCAACTCAACTGCTGTTAAATGC-3′) and Seq2 reverse (5′-TCTCTTCACTTTCTCTCAGGTTC-3′). Sequences were analyzed on the 3130xl Genetic Analyzer (Applied Biosystems).

RNA isolation from serum, RT-PCR, PCR amplification, molecular cloning of multiple PCR products and sequencing of env gp120 C2–C4

Env gp120 C2–C4 sequences from viral RNA in serum from donor and recipient were available from earlier studies (Rachinger et al, 2010, 2011). HIV-1 RNA isolation from serum samples, cDNA synthesis, molecular cloning of multiple PCR products and sequencing of env gp120 C2–C4 PCR products (549 nucleotides, corresponding to HXB2 envelope nucleotide positions 811–1290) was performed as described previously (Rachinger et al, 2010). From each time point a median of 3 C2–C4 env (range, 1–5) PCR products was generated and cloned. A median of 13 env gp120 C2–C4 env (range, 2–16) sequences was generated per time point (Table 1).

Prediction and determination of coreceptor use

The two study participants were routinely tested at approximately 3-monthly intervals for the presence of replication-competent CXCR4- using HIV-1 variants in PBMC using the MT-2 assay (Koot et al, 1992). CCR5 and CXCR4 coreceptor use of replication-competent clonal HIV-1 variants at the time points under study was determined by testing the ability of the virus to replicate in MT-2 cells (Van ’t Wout et al, 2008), U87.CD4.CCR5 cells, and U87.CD4.CXCR4 cells, as described previously (De Roda Husman et al, 1999). V3 sequence based prediction tools (Position Specific Scoring Matrix (PSSM)) (Jensen et al, 2003) and the geno2pheno coreceptor method (PR=5%) (Sing et al, 2007) were used to confirm the coreceptor use of the replication-competent clonal HIV-1 variants and to predict coreceptor use of sequences derived from viral RNA in serum.

Phylogenetic and molecular clock analysis

Nucleotide sequences were aligned using ClustalW (BioEdit v.7.0.9, Hall, 1999) and edited manually.

A Maximum Likelihood (ML) tree was reconstructed from published and unpublished env gp120 C2–C4 sequences from ACS participants, merged with an international panel of highly related, but not epidemiologically linked sequences (downloaded from the Los Alamos database). The best-fit nucleotide substitution model (TVM+1+G), selected by hierarchical likelihood tests (NLRTs, Modeltest v3.7, Posada and Crandall, 1998) was implemented in the heuristic search for the best ML tree applying NNI (nearest-neighbor-interchange) branch-swapping algorithm using PAUP*4.0 (Wilgenbusch and Swofford, 2003), starting with a Neighbor-Joining (NJ) tree constructed under the Hasegawa–Kishino–Yano (HKY85) model of evolution (Hasegawa et al, 1985). The resulting ML tree was rooted with the earliest sequence available and displayed with Dendroscope (Huson et al, 2007). Statistical support for nodes was generated using NJ bootstraping (1000 repeats).

Subsequently, ML and Bayesian MCMC analysis were performed on the alignment of env gp120 C1–V5 sequences derived from clonal HIV-1 variants from donor and recipient, and on a set of env gp120 C2–C4 sequences derived from viral RNA in serum aligned to the clonal HIV-1 variants but trimmed to the length of the C2–C4 region. ML phylogenies were reconstructed using PAUP*, applying the best-fit nucleotide substitution model and the TBR (tree-bisection-reconnection) branch-
swapping algorithm. The ML trees were rooted using one sequence of the earliest time point from the donor. Bayesian phylogenetic reconstruction was performed using Markov Chain Monte Carlo (MCMC) analysis implemented in BEAST v1.5.4 (Drummond and Rambaut, 2007). BEAST focuses on rooted, time-measured phylogenetic trees with a coalescent prior. We applied a general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites, a lognormal relaxed clock model (Drummond et al., 2006) and a flexible Bayesian skytree tree prior (Minin et al., 2008). MCMC chains were run sufficiently long (100 million generations) to ensure stationarity and adequate effective sample sizes as diagnosed using Tracer (http://tree.bio.ed.ac.uk/software/tracer/). Maximum clade credibility (MCC) trees were annotated using TreeAnnotator and visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

To compare evolutionary rates of viruses between donor and recipient, Bayesian MCMC analysis was performed on the env gp120 C1–V5 sequences from clonal HIV-1 variants for each patient separately. For the recipient, an additional analysis was performed solely on the clonal R5 variants sequences. BEAST MCMC analyses were run applying both a strict and a lognormal relaxed clock model and using the same substitution models as mentioned above (chain length 200 million); models were compared using Bayes factor testing (Suchard et al., 2003).

Selective pressure analysis

To quantify the differences in selective pressure between HIV-1 variants from donor and recipient, and between the R5 and CXCR4-using variants within the recipient, we estimated synonymous/non-synonymous substitution rate ratios (dN/dS) using codon substitution models implemented in the codeml program from the PAML package (Goldman and Yang, 1994; Yang, 1997). Specifically, we fitted different models that allow dN/dS to vary among lineages (Yang, 1998). Our comparison included a model that assumes a single dN/dS for lineages from donor and recipient (Model A), a model that specifies a different dN/dS for donor and recipient (Model B) and a model that allows a different dN/dS for the donor, the recipient’s R5 variant clade and the recipient’s CXCR4-using variant clade (Model C). Analyses were performed on env gp120 C1–V5 sequences derived from clonal HIV-1 variants and nested models were compared using the likelihood ratio test (Yang, 1998).

To investigate the accumulation of synonymous and non-synonymous substitutions over time, we also estimated branch lengths for the MCC phylogeny of donor and recipient based on env gp120 C1–V5 clonal HIV-1 variant sequences in expected synonymous (E[S]) and non-synonymous (E[N]) substitutions using a local codon model implemented in HyPhy (Kosakovsky Pond et al., 2009; Pond et al., 2005). We subsequently plotted E[S] and E[N] root-to-tip divergences using Patho-gen (http://tree.bio.ed.ac.uk/software/pathogen/).

Sensitivity of HIV-1 to anti-CCR5 monoclonal antibodies mediated inhibition in a PBMC-based assay

PBMC from 3 to 4 different healthy seronegative blood donors were isolated by Ficoll-isopaque density gradient centrifugation and stimulated for 3 days in Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) supplemented with 10% fetal calf serum (FCS; Hyclone), stimulated for 3 days in Iscove’s Modi in inhibition in a PBMC-based assay Sensitivity of HIV-1 to anti-CCR5 monoclonal antibodies mediated inhibition in a PBMC-based assay

PBMC from 3 to 4 different healthy seronegative blood donors were isolated by Ficoll-isopaque density gradient centrifugation and stimulated for 3 days in Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) supplemented with 10% fetal calf serum (FCS; Hyclone), 1 μg/ml phytohemaglutinin (PHA;Welcome), 100 U/ml penicillin and 100 μg/ml streptomycin (Pen/Strep;Gibco Brl), 5 μg/ml Ciproxin (Bayer) in a culture flask at a cell density of 5 × 10^6/ml. In a 96-well tissue culture plate, a final inoculum of 35 TCID50 (50% tissue culture in- fectious dose) was used to infect 1 × 10^6 PHA-stimulated PBMC previously incubated for 1 h at 37 °C with threefold serial dilutions of monoclonal antibody (mAb) RoAb13 or RoAb552 (highest concentration = 30 μg/ml) (Ji et al., 2007), kindly provided by Dr. Andreas Jeckle (Roche). Cultures were incubated for 1 week at 37 °C and 10% CO2. A median of 5 clonal HIV-1 variants (range, 2–6) were tested per time point (Table 1); each clonal HIV-1 variant was tested in triplicate. On day 7, a third of the culture was transferred to a new plate containing 1 × 10^5 new PHA-stimulated PBMC per well. Virus production in culture supernatants at days 7 and 11 was analyzed using an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (Tersmette et al., 1989). The percent inhibition was calculated by determining the reduction in p24 production in the presence of the antibody as compared to the cultures that lacked antibody. 50% inhibitory concentrations (IC50) were determined by linear regression.

Analysis of envelope molecular properties

Potential N-linked Glycosylation Sites (PNGS) were identified using N-Glycosite (Zhang et al., 2004) at the HIV database website (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). Charge was calculated by counting all charged amino acid residues per sequence, where R and K were counted as +1, H as +0.293, and D and E as −1.

Statistical analysis

Statistical analyses were performed in SPSS 16 software package. Longitudinal changes in sensitivity to anti-CCR5 MAbs RoAb13 and RoAb552, in charge and PSSM score of the gp120 C1–V5, were assessed using a Kruskal–Wallis test. Differences in sensitivity to anti-CCR5 MAbs RoAb13 and RoAb552, in charge and PSSM score of the gp120 C1–V5 and in number of PNGS in gp120 C1–V5, between two different time points were evaluated using the Mann–Whitney U test.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2011.10.005.

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References


Wilgenbusch, J.C., Swofford, D., 2003. Inferring evolutionary trees with PAUP* Chapter 6, Unit 6.4.