Survey of the temporal changes in HIV-1 replicative fitness in the Amsterdam Cohort

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Abstract

Changes in virulence and fitness during an epidemic are common among pathogens. Several studies have shown that HIV fitness increases within a patient during disease progression, while bottlenecks, such as sexual transmission, immune pressure and drug treatment can reduce fitness. In this study, we analyzed how these opposing forces have shaped HIV-1 fitness over time. Therefore, we compared the replicative fitness of HIV-1 isolates from newly infected untreated individuals, diagnosed for HIV-1 infection early in the AIDS epidemic in Amsterdam, the Netherlands, with more recent isolates. Twenty-five early and late HIV-1 isolates, carefully matched for seroconversion time, were competed head-to-head in a dual infection/competition assay, employing peripheral blood mononuclear cells. In contrast with previous studies, we observed a trend of increasing fitness over time in the HIV epidemic of Amsterdam. Apparently, the bottleneck, occurring with each transmission event, does not completely reset the fitness increase acquired during disease progression.

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Introduction

Fitness is a parameter describing the replicative capacity of an organism in a particular environment. In the case of HIV, fitness is affected by multiple factors, depending on the host environment (i.e., different target cells, immune responses, antiretroviral treatment), as well as host and viral genetics (Van Opijnen and Berkhout, 2005). Initial studies on attenuated HIV-1 variants with a deletion of the nef gene clearly indicated that reduced virus replication leads to delayed disease progression (Kirchhoff et al., 1995; Learmont et al., 1999; Dyer et al., 1999; Birch et al., 2001). Several studies over the past 5 years have suggested that the ability of HIV to replicate in vitro in primary human T cells, i.e., the majority of target cells during chronic HIV-1 infection, is related to pathogenicity and virulence (Quinones-Mateu et al., 2000; Troyer et al., 2005; Ariën et al., 2005a, 2005b).

Previous work has shown that, within an infected person, HIV-1 replicative fitness increases during progression of disease, suggesting that natural constraints such as the innate and adaptive human immune system are not restrictive enough to prevent increases in HIV-1 fitness (Quinones-Mateu et al., 2000; Troyer et al., 2005). Suboptimal antiretroviral treatment, giving rise to drug resistance mutations, may also influence fitness. Most primary drug resistance mutations result in a drop in replicative fitness that is subsequently compensated by secondary mutations, with a concomitant rebound in fitness (Harrigan et al., 1998; Martinez-Picado et al., 1999; Nijhuis et al., 1999; Weber et al., 2005). These observations indicate that drug-induced bottlenecks are not restrictive enough to permanently reduce the viral fitness. Recently, in vitro studies have shown that the replicative fitness of different HIV types and groups correlates with their prevalence in the human popula-
tion, i.e., HIV-1 group M > HIV-2 ≫ HIV-1 group O (Ariën et al., 2005a, 2005b; Ball et al., 2003). Furthermore, we and others presented data that suggests that the recombinant virus CRF02_AG has increased fitness compared with subtypes A and G which may underlie the increased spread of this recombinant in West Central Africa (Njai et al., 2006; Konings et al., 2006).

In a previous study, we have compared the replicative fitness of primary HIV-1 isolates from the beginning of the epidemic and more recent viruses from the ITM-clinic in Antwerpen, Belgium. Direct head-to-head competitions in activated peripheral blood T cells pointed to a significantly lower fitness of the more recent HIV-1 isolates (Ariën et al., 2005b; Quinones-Mateu, 2005). We suggested that serial genetic bottlenecks, occurring during transmission, could reduce viral fitness gradually, in accordance with in vitro observations by Yuste et al. (Yuste et al., 1999). However, a criticism on this study is that the viruses were not matched for seroconversion date and thus were not optimally controlled for duration of infection. Therefore, in the present study we have focused on the relative ex vivo fitness of primary HIV-1 isolates from patients within the first 9–18 months after infection and studied the change in HIV fitness over time. To this end, virus isolates from the beginning of the AIDS epidemic (1986) in Amsterdam, the Netherlands, were compared with more recent viruses (1996–2004) isolated in the same city. The virus collection of the Amsterdam Cohort Studies (ACS) of homosexual men is expected to be homogeneous with regard to subtype (only subtype B) and route of transmission (men who have sex with men; MSM) and is therefore very suitable for evolutionary analysis. Remarkably, and in contrast to the previous data (Yuste et al., 1999; Ariën et al., 2005b), our results suggest that HIV-1 may have evolved to become more fit over time in Amsterdam.

Results

Characterization of early and late HIV-1 isolates

“Early” (1986) and “late” (1996–2003) samples were selected from HIV-1 infected MSM with known seroconversion date and without antiretroviral treatment (Table 1). Viruses were propagated from frozen PBMC in short-term coculture with activated seronegative donor PBMC. PBMC from one donor were used to propagate all samples while a second PBMC donor was used to titre the virus stocks and perform dual infections/competitions. The time since seroconversion of the “early” (n = 14) and “late” (n = 11) viruses was similar (347 versus 354 days, respectively; p = 0.89, Mann–Whitney test). Patients

Table 1

Virus characteristics

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<tr>
<th>Virus ID</th>
<th>Sample date</th>
<th>TSSc</th>
<th>CD4</th>
<th>Plasma VL</th>
<th>Coreceptor</th>
<th>Subtype</th>
<th>Age</th>
<th>Origin</th>
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</table>

“Early” viruses were isolated in 1986; “late” viruses originate from the period between 1995 and 2004; TSSc: time since seroconversion (in days); CD4+ T cell count (in x 10^9 cells per litre blood); viral load (in Log10 plasma RNA copies/ml); NL: The Netherlands; ES: Spain; PT: Portugal; SG: Singapore; VE: Venezuela; FR: France; UK: United Kingdom.
Comparing the replicative fitness of early and late HIV-1 isolates in PBMC

All 25 HIV-1 isolates were competed (~300 dual infections in total) against each other in activated human PBMC from one donor. We have previously shown that although total virus production can differ significantly in PBMC of different donors, the relative production of each virus (i.e., relative fitness) in dual infections remains similar with different donors (Quiñones-Mateu et al., 2000; Marozsan et al., 2005; Troyer et al., 2005; Ariën et al., 2005a, 2005b, 2006). The initial viral inoculum (5 × 10^4 infectious particles per cell) was removed 24 h after infection and de novo virus production was monitored by HIV-1 capsid (p24) antigen detection in cell culture supernatant. Upon peak virus production, viral DNA was extracted and subsequently PCR amplified and submitted to heteroduplex tracking assays (HTA) to analyze the virus mixture. Single virus infections were performed to provide control samples for HTA analysis. Relative fitness was calculated as described in materials and methods (Quinones-Mateu et al., 2000).

In a first, more general approach, we analyzed the mean relative fitness of each virus (from competitions against all other 24 viruses), both “early” and “late”. A higher mean replicative fitness was observed for the “late” viruses (W_{1986}=0.77 and W_{1996–2004}=1.33; p<0.04, paired t test). Fig. 2A shows a ranking of all viruses according to their mean relative fitness. Closer examination showed that the same trend occurred in inter-group fitness, i.e., the competitions of each virus from the “late” group against all the viruses from the “early” group and vice versa. The “late” HIV-1 isolates outcompeted the “early” viruses in 116 out of 144 competitions (80.6%). The mean relative fitness (W) of all “early” HIV-1 strains was significantly lower than that of “late” HIV-1 isolates (inter-group W_{1986}=0.47 and W_{1996–2004}=1.53; p<0.01, paired t test) (Fig. 2B). Direct competitions between isolates within the same group (intra-group fitness) showed that individual differences in fitness exist but that the average fitness in each group is ∼1, i.e., not different (Fig. 2B). Finally, Spearman rho test revealed no significant correlations between the relative fitness and CD4+ T cell count and between the relative fitness and time since seroconversion.

Discussion

Two evolutionary phenomena (The Red Queen and Müller’s Ratchet) determine viral fitness within an infected individual and in the epidemic. According to the “Red Queen” hypothesis (Van Valen, 1974; Clarke et al., 1994), fitness will increase if several distinct viruses (e.g., the different members of the quasi-species within a patient) compete with each other in an ever expanding population and in the absence of any stringent selection pressure. This appears to be the case in the chronic phase of an untreated infection with HIV, which is characterized by extensive viral replication in activated CD4+ T cells in the absence of strong immune or drug pressure. In accordance with this hypothesis,

Fig. 1. Phylogenetic tree, based on Pro-RT nucleotide sequences of all virus samples, showing the genetic distances between “early” and “late” HIV-1 isolates. Although there is more sequence heterogeneity in the “late” samples, particular clusters in the “early” or “late” samples are not observed.
we showed that the *ex vivo* fitness of a patients’ virus strongly correlates with plasma RNA load and with disease progression *in vivo* (Quinones-Mateu et al., 2000; Troyer et al., 2005). Moreover, we also found that intra-patient HIV-1 replicative fitness increases progressively in function of time after seroconversion and correlates with increasing HIV-1 *env* genetic diversity (Troyer et al., 2005).

On the contrary, serial bottlenecks result in a decrease of the quasi-species heterogeneity and viral fitness according to “Müllers Ratchet” principle (Müller, 1964; Chao, 1990). Such
bottlenecks may be induced by strong immune and drug pressure, but they may also occur during inter-person transmission events. The “transmission bottleneck” is deduced from the observation that the quasi-species in the newly infected subject is more homogeneous as compared to that in the source subject, indicating that only a limited number of clones (or genotypes) are transmitted (Duarte et al., 1992; Escarmis et al., 1996).

In this context, we previously compared the replicative fitness of primary HIV-1 isolates from the beginning of the epidemic and more recent viruses from the ITM-clinic in Antwerpen, Belgium (Ariën et al., 2005a, 2005b; Quinones-Mateu, 2005). Competing HIV-1 isolates from the late eighties and isolates from 2002 to 2003 revealed that the more recent HIV-1 isolates replicated significantly less efficiently. The Antwerp population, however, consisted of heterogeneous hosts (men and women of diverse ethnicities, different routes of transmission and durations of infection) and viruses (diverse subtypes, tropism). Moreover, the time since seroconversion was unknown and patients in all stages of disease were represented. To overcome the possible impact of heterogeneity, the samples from both groups were carefully matched for CD4+ T cell count, viral load and viral subtype (Ariën et al., 2005b).

Remarkably, the current data on the Amsterdam Cohort suggest a trend towards increased replicative fitness over almost the same period in time in another West-European city. Although both studies were performed with the same techniques by the same team in the same laboratory, there are various differences in patient and viral characteristics, which might explain the different outcome. In stark contrast to the Antwerp cohort, the Amsterdam Cohort is much more homogeneous, i.e., exclusively subtype B viruses from MSM living in Amsterdam. Furthermore, the viruses from Amsterdam were isolated early after infection (typically between 9 and 18 months) and early and late groups were primarily matched for time since seroconversion. Given that the “early” and “late” viruses were isolated at a time point when viral evolution within each patient was still limited, the observed fitness differences between the two comparable and homogenous patients groups, “early” and “late” in the epidemic, are likely to reflect the effect of the transmission bottleneck over time.

Recently, Muller et al. (2006) found a lack of change in HIV virulence over time, as assessed by a number of clinical markers, i.e., the slope of CD4+ T cell count, CD4:CD8 ratio and viral set point. This lack of long-term evolution of virulence markers and the presence of patients with either slow or rapid progression during the observation period suggests that both highly virulent and attenuated strains had spread in the population. Obviously, the set-up of the Swiss study differs fundamentally from the current study. Muller et al. measured the outcome of the virus–host interaction in clinical terms and make broad indirect conclusions on evolution of “virulence”. In the current study, we have focused on the replicative fitness as an ex vivo parameter that measures an intrinsic viral characteristic, that is the result of viral-host interactions, and that may constitute one of the determinants of overall (clinical) virulence.

Because of the conflicting results on the evolution of HIV fitness presented in the few studies carried out today and the uncertainty of the actual effect of transmission on viral fitness, it is advisable to design larger studies using samples from various epidemiological settings and that are well-controlled for host and viral factors (such as mode of transmission, viral subtype, ethnic background of the patients etc.). Preferentially, samples from seroconverters should be considered for this kind of evolutionary analysis. Unfortunately, it may be challenging to perform these kind of studies on larger scale because well-documented HIV-1 seroconversion samples from the early years of the epidemic might be difficult to find.

Materials and methods

Cells

Peripheral blood mononuclear cells (PBMC), obtained from an HIV-seronegative buffy coat by Ficoll-Hypaque density gradient centrifugation, were stimulated with 2 μg/ml phytohemagglutinin (PHA) for 3 days and further maintained in RPMI 1640-2 mM L-glutamine medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 ng/ml interleukin-2 (IL-2), 100 U/ml penicillin and 100 μg/ml streptomycin, further referred to as complete medium. The resulting cell population consists of >90% PHA/IL-2 T blasts.

Patients and viruses

Twenty-five HIV-1-positive MSM (men who have sex with men) participating in the Amsterdam Cohort Studies were selected based on the availability of stored infected PBMC, isolated early after seroconversion. Virus was propagated starting from cells isolated from fourteen subjects that seroconverted in the mid eighties (early group) and from eleven in the late nineties (late group), in short-term cocultures with HIV-seronegative donor PBMC. Plasma viral load of each patient was determined with the NASBA assay (Organon) (Table 1). The coreceptor tropism was determined on U87.CD4 cells expressing either CCR5 or CXCR4 (Table 1). Virus titers were determined in PHA/IL-2 activated T cells from one HIV-negative donor. Virus stocks were serially diluted in six-fold replicates and then added to activated T cells to measure TCID50. Following 10 days of incubation, supernatant was harvested and virus production was measured using an in-house p24 antigen capture ELISA. Tissue culture dose for 50% infectivity (TCID50) was calculated using the Reed and Muench (1938) method.

Growth competition assays

Dual infections/competitions were performed in duplicate with twenty-five primary HIV-1 isolates in PHA/IL-2 activated T cells from one donor (same donor as for TCID50 determination and same blood draw) as described earlier (Quinones-Mateu et al., 2000; Marozsan et al., 2005; Troyer et al., 2005; Ariën et al., 2005a, 2005b, 2006). Activated T cells were
infected with two viruses at equal multiplicity of infection (0.0005 MOI). Uninfected cultures were used as HIV-negative controls and monoinfected cell cultures of each virus correspond to positive controls. The estimated frequency of recombination between HIV-1 isolates in the dual infection competition assay is 1% of the dual virus production and well below the limit of HTA detection (Quinones-Mateu et al., 2000). Virus mixtures were incubated with $2 \times 10^5$ PBMC at 37 °C in 5% CO$_2$ and washed three times with 1× phosphate-buffered saline (PBS) 24 h post infection and then resuspended in complete medium (Ariën et al., 2005a, 2005b, 2006; Njai et al., 2006). Cell-free supernatant was assayed for p24 antigen capture ELISA (Beirnaert et al., 1998). Two detection 7 and 10 days post infection with an in house HIV-1 p24 antigen capture ELISA (Beirnaert et al., 1998). Two aliquots of supernatant and cells were harvested at day 10 after infection and stored at −80 °C for subsequent analysis.

**Heteroduplex tracking assay (HTA)**

Proviral DNA was extracted from lysed PBMC using the QIAamp DNA Blood kit (Qiagen, GmbH, Belgium). HIV-1 DNA was PCR amplified using a set of external primers (envB: 5′-AGAAAGAGCAGAAGACGATGGCAATGAC-3′ and ED14: 5′-TCTTGCCCTGGAGCTTTGATGCCCAGAC-3′), followed by nested amplification (E80: 5′-CCATTTCCA-TACATTATTG-3′ and E125: 5′-CAATTTCGTGGTCCTCC-CTCTGAGG-3′). The nested PCR reaction was carried out in a 100-μl reaction mixture under defined cycling conditions (Quinones-Mateu et al., 2000; Ariën et al., 2005a). Nested PCR products from env (C2V3) were analyzed by heteroduplex tracking assays (HTA) to determine the amount of virus production in the dual infection/competition experiments, as described previously (Quinones-Mateu et al., 2000; Ariën et al., 2005a, 2005b, 2006; Njai et al., 2006). Radiolabeled DNA probes were PCR amplified from regions of env using the same primer sets described above, and one of the nested primers was radiolabeled using T4 polynucleotide kinase and 2 μCi of [γ-32P] ATP (Quinones-Mateu et al., 2000).

Competitions were analyzed with two different probes derived from primary HIV-1 isolates unrelated to this study (a subtype A probe: A$_1$ and a subtype E probe: E$_3$). In previous studies using diverse HIV-1 subtypes, we have shown that probe binding and specificity does not significantly differ among HIV-1 M subtypes (Ariën et al., 2005a). Subsequently, radiolabeled probes were separated on 1% agarose gels and purified using the QIAquick gel extraction kit (Qiagen, GmbH, Belgium). HTA reaction mixtures contained DNA annealing buffer (100 mM NaCl, 10 mM Tris–HCl [pH 7.8], 2 mM EDTA), 10 μl of amplified DNA from the competition culture and 0.1 pmol of radioactive probe DNA were denatured at 95 °C for 3 min followed by incubation at 37 °C for 5 min and rapid transfer on wet ice to allow re-annealing. DNA heteroduplexes were resolved on 5% TBE non-denaturing polyacrylamide gels (BIORAD, Belgium) for 1 h 15 min at 200 V. Gels were dried for 45 min at 80 °C, exposed and scanned with a phosphor imager (Cyclone®, PerkinElmer, Boston, USA) and analyzed with OptiQuant (PerkinElmer, Boston, USA).

**Estimation of viral fitness**

The final ratio of two viruses produced in a dual infection was measured by heteroduplex tracking analysis and compared to the production in monoinfections. Production of individual HIV isolates in a dual infection (fo) was divided by the initial proportion in the inoculum (Io). This parameter is referred to as relative fitness ($W = fo / io$) (Quinones-Mateu et al., 2000).

**Sequencing**

The complete HIV-1 protease (Pro) gene and the first 335 codons of the reverse transcriptase (RT) gene were sequenced. The sequences were generated in the AMC diagnostic unit with the ViroSeq HIV-1 genotyping kit version 2 (Celera Diagnostics, Alamada, CA, USA). Electrophoresis and data collection were performed on an ABI PRISM 3730 genetic analyser (Applied Biosystems, Foster City, CA, USA).

NJ (Neighbor-Joining) trees based upon Kimura 2-parameter distance matrices were constructed using the MEGA software package (www.megasoftware.net) and 900 bootstrap replicates were analyzed.

**Statistical analyses**

Analyses were conducted with Spearman rho, Mann–Whitney U and Student’s t tests (SPSS v. 12.0 for Windows) (SPSS Inc., Chicago, IL, U.S.) to calculate correlations and significance levels.

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