In vitro replication capacity of HIV-2 variants from long-term aviremic individuals

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Received 9 March 2006; returned to author for revision 27 March 2006; accepted 25 May 2006
Available online 30 June 2006

Abstract

To establish whether efficient suppression of virus replication in HIV-2-infected individuals is associated with low replicative capacity of HIV-2, replication kinetics of HIV-2 variants from long-term aviremic individuals was analyzed and compared with that of the relatively slow-replicating HIV-1 variants from asymptomatics and long-term nonprogressors (AS/LTNP). On average, HIV-2 from aviremic individuals had lower replication rates than HIV-1 variants from AS/LTNP in cells of 8 donors (0.45 log10 [range 0.14–0.77] vs. 0.58 log10 [range 0.32–0.99] pg RT/ml/day, P = 0.036). The relatively low replication rate of HIV-2 compared to HIV-1 variants was not related to different sensitivities to inhibition by CD8+ T cells or different degrees of infectivity. HIV-2 replication rates increased with progressive infection and with switch from CCR5 to CXCR4 usage.

The relatively low replicative capacity of HIV-2 variants from aviremic individuals likely contributes to the low viral load and benign course of infection in these individuals.

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Keywords: HIV-2; Biological clone; Replication rate; Aviremia; Long-term nonprogressor; Pathogenesis

Introduction

Although both HIV-1 and HIV-2 infection cause AIDS in humans, HIV-2-infected individuals generally remain AIDS-free for longer periods and have lower mortality rates than HIV-1-infected individuals (Whittle et al., 1994; Marlink et al., 1994; Marlink, 1996; Ricard et al., 1994; Poulsen et al., 1997; Berry et al., 2002). In fact, the relatively high prevalence of HIV-2 in older people and a mortality rate in these individuals that is similar to that of uninfected individuals suggest that although some HIV-2-infected individuals progress to AIDS at a similar rate as HIV-1-infected individuals, many may never develop AIDS during their lifetime (Poulsen et al., 1989, 1997, 2000; Larsen et al., 1998; Ricard et al., 1994). During asymptomatic HIV-2 infection, plasma loads are often too low to be detected using sensitive RT-PCR techniques, which contrasts with asymptomatic HIV-1 infection, where even long-term nonprogressors (LTNP) often have low but detectable plasma loads (Berry et al., 1998; Gottlieb et al., 2002; Popper et al., 1999; Damond et al., 2002; Cao et al., 1995; Barker et al., 1998; Blaak et al., 1998; Rodes et al., 2004). We have previously observed that also the infectious PBMC load is very low in aviremic HIV-2-infected individuals, in a range comparable to that in HIV-1-infected individuals treated with HAART, and similar to only a small minority of asymptotically HIV-1-infected individuals (Blaak et al., 2004). These findings suggest a qualitative

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0042-6822/ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.05.029
difference in the degree of virus control in asymptomatic HIV-1 and HIV-2 infection. Moreover, while a high level of control and long-term nonprogression appears common in HIV-2 infection, it is exceptional in HIV-1 infection.

In HIV-1 infection, the level of virus control is in part attributable to the efficiency of the triggered immune response (Sewell et al., 2000; Xiao et al., 2002). Secondly, it is correlated with the ex vivo replication capacity of HIV-1 variants (Connor et al., 1993; Asjo et al., 1986; Connor and Ho, 1994; Blaak et al., 1998; Campbell et al., 2003; Quinones-Mateu et al., 2000). Although a high replicative capacity is often associated with the ‘SI’ or CXCR4-using phenotype, differences in in vitro replicative capacity were also observed between CCR5-restricted HIV-1 variants from LTNP and individuals who develop AIDS, and between individuals with low or high viral load (Blaak et al., 1998; Campbell et al., 2003; Quinones-Mateu et al., 2000), demonstrating that replicative capacity can evolve independently of coreceptor usage (Blaak et al., 1998; Connor and Ho, 1994).

The main objective of this study was to establish whether the relatively high level of virus control in HIV-2-infected individuals is associated with a relatively low replicative capacity of the infecting variants. For this purpose, we have compared replication kinetics of HIV-2 variants isolated from untreated, long-term aviremic individuals with that of the – for HIV-1 standards – relatively slow HIV-1 variants from LTNP (Blaak et al., 1998). Because previous studies had demonstrated the existence of different HIV-2 replication phenotypes in PBMC and cell lines and suggested an association with stage of disease (Albert et al., 1990; Castro et al., 1990), we also determined whether HIV-2 variants from individuals who control infection have different replicative capacity in PBMC than HIV-2 variants from individuals who progress to AIDS.

**Results**

**Subjects and time points of analysis**

Of 11 asymptomatic HIV-1 and HIV-2-infected individuals included, 10 had a known seropositive follow-up of 7.3 to 12.6 years with high and stable CD4 counts and were termed long-term nonprogressors (LTNP) (Table 1). At the moment of virus isolation, the CD4+ T cell numbers of HIV-1 AS/LTNP were slightly higher than that of the HIV-2 LTNP (average 1003 vs. 758 cells per μl of blood respectively; P = 0.2, Mann-Whitney U). Four of 6 HIV-1-infected AS/LTNP and all 5 HIV-2-infected LTNP had undetectable plasma load. The HIV-2

**Table 1**

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>CD4 (/μl)</th>
<th>RNA (/ml)</th>
<th>PBMC load (IUPM/d)</th>
<th>AS follow-up (months/years)</th>
<th>Disease stage</th>
<th>CCR5 and CXCR4 usage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HIV-2 AS/LTNP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RH2-3 CVI A 96</td>
<td>770</td>
<td>&lt;500*</td>
<td>0.2</td>
<td>151/12.6</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-8 GH B 80</td>
<td>900</td>
<td>&lt;500</td>
<td>0.01</td>
<td>134/11.1</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-13 CVI A 52</td>
<td>900</td>
<td>&lt;500</td>
<td>0.6</td>
<td>112/9.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-14 CVI A 51</td>
<td>550</td>
<td>&lt;500</td>
<td>0.3</td>
<td>100/8.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-22 CVI B 30</td>
<td>670</td>
<td>&lt;500</td>
<td>0.9</td>
<td>87/7.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td><strong>HIV-1 AS/LTNP</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH1-1 NIG A 70</td>
<td>1150*</td>
<td>&lt;500*</td>
<td>10</td>
<td>112/9.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>RH1-2 GAM A/G 41</td>
<td>760*</td>
<td>24,600*</td>
<td>3</td>
<td>78/6.5</td>
<td>AS</td>
<td>R5</td>
</tr>
<tr>
<td>ACH78 NL B 17</td>
<td>750</td>
<td>&lt;1000</td>
<td>2</td>
<td>124/10.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>ACH441 NL B 16</td>
<td>1110</td>
<td>&lt;1000</td>
<td>1</td>
<td>149/12.4</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>ACH583 NL B 24</td>
<td>830</td>
<td>&lt;1000</td>
<td>1</td>
<td>138/11.5</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td>ACH750 NL B 16</td>
<td>1420</td>
<td>19,000</td>
<td>16</td>
<td>148/12.3</td>
<td>LTNP</td>
<td>R5</td>
</tr>
<tr>
<td><strong>HIV-2 progressors</strong></td>
<td></td>
<td></td>
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<tr>
<td>RH2-1 CVI A 57</td>
<td>240</td>
<td>NT</td>
<td>36</td>
<td>NA</td>
<td>PROG</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-5 CVI A 38</td>
<td>120</td>
<td>110,000</td>
<td>10</td>
<td>NA</td>
<td>PROG</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-7 CVI A 33</td>
<td>10</td>
<td>+</td>
<td>673</td>
<td>NA</td>
<td>PROG</td>
<td>R5</td>
</tr>
<tr>
<td>RH2-21 CVI A 1</td>
<td>60*</td>
<td>59,000</td>
<td>1</td>
<td>NA</td>
<td>PROG</td>
<td>R5/X4</td>
</tr>
<tr>
<td>RH2-24 CVI A 4</td>
<td>70*</td>
<td>23,000</td>
<td>63</td>
<td>NA</td>
<td>PROG</td>
<td>R5/X4</td>
</tr>
<tr>
<td>RH2-26 NL B 1</td>
<td>10</td>
<td>330,000</td>
<td>71</td>
<td>NA</td>
<td>PROG</td>
<td>R5/X4</td>
</tr>
</tbody>
</table>


*b Time between first visit to the clinic and the sample of virus isolation.

*c CD4 numbers and RNA copies at the time point of virus isolation, when unavailable data from the next time point were taken (*). The detection limit of the RT-PCR used for load determination at the moment of virus isolation was 500 copies ml$^{-1}$ for the HIV-2-infected individuals and RH1-1 and RH1-2, and 1000 copies ml$^{-1}$ for the Amsterdam Cohort (ACH) participants. NT = not tested, + = positive, not quantified.

*d Infectious Units per Million CD4 T cells.

*e Length of known asymptomatic follow-up, NA = not applicable.

*f LTNP = long-term nonprogressor, AS = asymptomatic individual, PROG = progressor.

*g CCR5 and CXCR4 usage of infecting variants based on results with CCR5$^{-/}$ PBMC and GHOST or U87 cells. R5 = individuals with CCR5-using variants only, R5/X4 = individuals with co-existing CCR5-using and CXCR4-using variants.
LTNP had a lower infectious load (0.4 vs. 5.5 IUPM; $P = 0.006$, Mann-Whitney U). The lower viral load of the HIV-2-infected LTNP, even though the moment of analysis was on average from temporally more advanced stages (62 vs. 31 months), reflects the better control of virus replication in asymptomatic HIV-2 vs. HIV-1 infection (Popper et al., 1999; Berry et al., 1998; Gottlieb et al., 2002). HIV-2 progressors all had high virus loads and/or low CD4 counts, and from 3 out of 6 CXCR4-using variants were isolated (Table 1).

Replication kinetics of HIV-2 variants from aviremic LTNP vs. HIV-2 variants from progressors

Replication kinetics of HIV-2 variants from aviremic LTNP – all CCR5-restricted in PBMC (‘R5’ variants) – were compared with R5 as well as CXCR4-restricted (X4) variants from progressors. As a measure for replication, we determined the accumulation of functional RT enzyme concentrations in culture supernatants, reflecting newly synthesized virus particles (Fig. 1a). Most variants had an S-shaped growth curve, with a period of optimal growth preceded by a ‘lag-phase’ and followed by a plateau-phase (Fig. 1a). The ‘lag-phase’ reflects the time needed to reach the cut-off value of the assay. Because any increase in supernatant RT during this phase is relative to the cut-off value, slopes underestimate true replication rates. The most rapid-replicating variants, particularly X4 variants, did not have a ‘lag-phase’. The plateau-phase is characterized by gradually declining slopes, presumably due to exhaustion and/or reduced infectability of target cells (Campbell et al., 2003). For all three groups of viruses, optimal growth was observed between days 3 and 5 (Fig. 1b). At this point, i.e., before growth started to decelerate to various degrees for the different types of viruses, the average level of RT accumulated was 0.74 log_{10} higher for the R5 progressor than LTNP variants (1446 vs. 263 pg RT/ml; $P = 0.04$, T test), indicating a higher replication rate. X4 variants had accumulated even higher levels, on average 1.4 log_{10} higher than LTNP variants (6794 pg RT/ml; $P = 0.005$, Mann-Whitney U).

In contrast to X4 variants that all replicated rapidly, the R5 progressor variants were heterogeneous with respect to replication phenotypes, ranging from variants with kinetics similar to that of the LTNP variants to those nearly as rapid as that of X4 variants (Fig. 1a). In none of the groups specific differences between subtype A and B viruses were observed.

Replication kinetics of HIV-2 variants from LTNP vs. HIV-1 variants from AS/LTNP

To determine how replication kinetics of HIV-2 variants from aviremic LTNP compares to that of the relatively slow-replicating HIV-1 variants from AS/LTNP, we analyzed three early stage subtype B HIV-1 variants from LTNP with typically slow/intermediate phenotypes as revealed in our previous study (Blaak et al., 1998). Subtype A and A/G HIV-1 variants from AS/LTNP of West-African origin were additionally included, to control for differences related to replication in hosts of different ethnic origin.

Replication kinetics of subtype B and subtype A and A/G AS/LTNP variants were similar in PBMC of 3 donors tested (Fig. 2a). The relatively slow replication kinetics of these variants for HIV-1 standards was confirmed by using as a reference strain a R5 progressor variant with a previously identified rapid replicating phenotype characteristic for late stage HIV-1 (Blaak et al., 1998). On average, HIV-2 variants from aviremic individuals had even lower replication kinetics than the AS/LTNP HIV-1 variants (Fig. 2b). At the last time point where growth rates were still optimal for both types of HIV, HIV-1 RT concentrations were 0.76 log_{10} (donor A, day 5), 0.58 log_{10} (donor B, day 7) and 0.99 log_{10} (donor C, day 11) higher than those of HIV-2.

Fig. 1. Replication kinetics of HIV-2 variants from LTNP vs. HIV-2 from progressors. Accumulation of functional RT concentrations in culture supernatants of acutely infected PBMC consisting of a mix of cells from 5 donors during 14 days of culture. (a) Shown are individual growth curves of R5 variants from LTNP (left), R5 variants from progressors (middle) and X4 variants from progressors (right). Viruses of LTNP RH2-8 and RH2-22, and progressor RH2-26 are of subtype B, all other viruses are of subtype A. Dashed lines represent the cut-off value of the RT assay. (b) Average growth curves for the three types of variants. Error bars represent standard error of the mean. Arrow designates latest time point of optimal growth, asterisk indicates statistical significance at the $P < 0.05$ level between R5 variants from LTNP and progressors (Student’s T test).
Replication rates of HIV-1 and HIV-2 variants

For each HIV variant and donor combination from the experiment shown in Fig. 2, replication rates were calculated based on the optimal increases in RT, assuming these represent ‘true’ growth rates, i.e., when target cell availability nor infectability are rate limiting (Figs. 3a, b). HIV-2 and HIV-1 AS/LTNP growth rates were on average 0.44 log_{10} vs. 0.58 log_{10} pg RT/ml per day, 0.33 log_{10} vs. 0.44 log_{10} pg RT/ml per day and 0.14 log_{10} vs. 0.32 log_{10} pg RT/ml per day, for donors A, B and C respectively (Fig. 3b). When continuously produced at these rates for 13 days HIV-1 AS/LTNP variants will accumulate 1.4 log_{10} (donor B) to 2.6 log_{10} (donors A and C) higher amounts of virus than the HIV-2 variants (Fig. 3c). Of note, this average HIV-2 LTNP growth rate was very similar to that observed in cells of donor A (0.44 and 0.46 log_{10}; compare Figs. 3c and d), suggesting similar replication rates for HIV-1 AS/LTNP (Fig. 3c) and HIV-2 R5 progressor (Fig. 3d) variants as well (0.58 log_{10} and 0.57 log_{10}, respectively). In support of this, HIV-1 AS/LTNP and R5 HIV-2 progressor viruses had similar levels of RT levels accumulated 7 days p.i. when directly compared in an independent experiment (not shown).

Lower replication rate of HIV-2 vs. HIV-1 AS/LTNP is not determined by CD8+ T cells

CD8+ T cells produce numerous factors that influence HIV-1 replication (reviewed by Tomaras and Greenberg (2001)). To rule out the possibility that the observed differences between HIV-1 and HIV-2 replication rates were explained by different sensitivities to CD8+ T cell factors, we compared HIV-1 and HIV-2 replication rates in PBMC from which CD8+ T cells were removed (Fig. 4a). The differences between HIV-1 and HIV-2 were in the same range as observed earlier, although P values were higher (0.06–0.14) likely due to smaller group sizes. Differences were statistically significant when results in three donors were combined (0.57 vs. 0.79 log_{10} pg/ml/day,
The lower replication rates of HIV-2 variants in the absence of CD8+ T cells demonstrate that the slower replication of HIV-2 variants is not explained by a higher sensitivity to antiviral activity of CD8+ T cells. In fact, depletion of CD8+ T cells had a slightly larger effect on HIV-1 than on HIV-2 replication (Fig. 4b).

In whole PBMC as well as CD8-depleted PBMC of most donors, the HIV-1 AS/LTN variants displayed a broader range of growth rates compared to HIV-2.
of replication rates than the HIV-2 variants (Figs. 3b and 4a). Importantly, however, the majority of HIV-2 variants had growth rates as low as, and often even lower than, the slowest of HIV-1 AS/LTNP variants.

**Difference in HIV-2 LTNP and HIV-1 AS/LTNP replication rates is not determined by differences in infectivity**

In infection experiments, HIV-1 and HIV-2 inocula had been equalized for functional RT content in virus stocks, which reflects infectious particle content but still allows for differences in infectivity per ‘RT unit’ or infectious particle. To determine whether the lower replication kinetics of HIV-2 variants was explained by lower infectivity at equal amounts of functional RT, we estimated the frequency of cells infected with replication-competent virus (i.e., infectious units) after exposure and determined replication kinetics in parallel cultures of the same cells. Despite the lower replication rates of the HIV-2 compared to HIV-1 variants (Fig. 4a), average frequencies of infectious units after 1 round of infection were similar (Fig. 5a), demonstrating similar degrees of infectivity and suggesting different levels of virus produced per productively infected for HIV-1 and HIV-2.

Single cycle infectivity of the HIV-1 and HIV-2 variants was additionally determined using GHOST-CCR5 cells. After one round of replication, the percentages of GFP-expressing, or actively virus-replicating cells, were comparable for HIV-1 and HIV-2 variants (Fig. 5b), confirming the results observed in PBMC. The kinetics of GFP expression were slightly lower in HIV-2-infected cells however, as evidenced by a delay in the detection of GFP+ cells in the HIV-2-exposed cells (0.3% vs. 0.7% positive cells 14 h p.i.) and a slower increase in mean
fluorescence intensity (MFI) in GFP+ cells during the first 17 h of infection (Fig. 5c). Since it is highly unlikely that re-
infection can be detected during the first 26 h p.i., the observed increases in MFI and numbers of GFP-positive cells probably reflect an increase in the levels of GFP expressed per infected cell and the number of cells starting to produce GFP above detection limits respectively, thus indicating a difference in the kinetics of protein synthesis between HIV-1- and HIV-2-
infected cells.

Discussion

The present study is the first to characterize replication properties of HIV-2 variants isolated from healthy infected individuals who maintain low, undetectable plasma load for prolonged periods. The study is also the first to compare the replication kinetics of individual biological HIV-2 clones – as opposed to bulk isolates – from different stages of infection as well as biological HIV-1 clones.

We have shown that long-term healthy aviremic individuals carry HIV-2 variants that have lower replication rates than variants from individuals who progress to AIDS, demonstrating an association between in vitro replication capacity and disease progression reminiscent of what is known for HIV-1 (Connor et al., 1993; Asjo et al., 1986; Connor and Ho, 1994; Blaak et al., 1998; Campbell et al., 2003; Quinones-Mateu et al., 2000). Especially CXCR4 usage was strongly associated with high replication rates, while CCR5-using variants were more heterogeneous with respect to replicative capacity. Of note, 2 of the progressor variants with relatively slow replicative capacity were isolated from an individual who was treated with AZT and 3TC at the moment of virus isolation (RH2-5). Both variants contained a Q151M and M184V mutation in RT (not shown) which are associated with AZT and 3TC resistance respectively (Colson et al., 2005; Descamps et al., 2004; Brandin et al., 2003; van der Ende et al., 2000), and it cannot be excluded that in this case the low replication capacity is related to reduced fitness associated with these mutations.

HIV-2 variants from LTNP were compared with the relatively slow-replicating HIV-1 variants from AS/LTNP in cells of 8 different donors, resulting in average rates of 0.45 log10 (range 0.14–0.77) and 0.58 log10 pg RT/ml/day (range 0.32–0.99) respectively. In comparison, the average replication rate in cells of the same donors of the rapid HIV-1 reference strain was 0.98 log10 (range 0.71–1.32), indicating that the difference between HIV-1 and HIV-2 from AS/LTNP is more subtle than that between HIV-1 from LTNP and progressors. The magnitude of the difference between HIV-2 and HIV-1 from AS/LTNP varied between donors, and as an exception to a rule, HIV-1 AS/LTNP replicated less efficiently than HIV-2 LTNP variants in cells of one donor (not shown). This suggests the existence of specific
host conditions that favor the replication of HIV-2 variants to that of HIV-1 AS/LTNP variants that are more replication competent under most conditions. The nature of responsible host factor(s) is not known but may be linked to our observation that replication of HIV-1 variants was more affected by depletion of CD8+ T cells than that of HIV-2 variants, suggestive of a higher sensitivity to (one or more of) the virus inhibitory factors that are produced by CD8+ T cells (reviewed by Tomaras and Greenberg, 2001).

The low replicative capacity of HIV-2 variants from long-term aviremic individuals compared to HIV-1 variants from AS and LTNP has two implications. Firstly, since HIV-1 from LTNP have relatively low replicative capacity for HIV-1 standards (Blaak et al., 1998; Quinones-Mateu et al., 2000), the finding illustrates the low replicative capacity of these HIV-2 variants relative to HIV-1 in general. Principally, most of the HIV-2 variants had replication rates comparable to, or even lower than, the slowest viruses among the HIV-1 variants presently studied. One of these slow-replicating HIV-1 variants, isolated from subject ACH78, was also among the slowest in our previous study, which included 105 different HIV-1 variants from LTNP and progressors (Blaak et al., 1998), suggesting this variant may represent the slow extremity of naturally occurring HIV replication phenotypes. Secondly, our findings suggest that subtle differences in replicative capacity between HIV-1 and HIV-2 variants may contribute to the different degrees of control of virus replication generally observed between HIV-2 LTNP and HIV-1 LTNP (Cao et al., 1995; Barker et al., 1998; Blaak et al., 1998; Rodes et al., 2004).

We did not observe differences in infectivity between HIV-1 and HIV-2, suggesting both types of HIV have similar entry efficiencies, although we cannot rule out that polybrene present in culture medium differentially affects the entry of different viruses. Nevertheless, our results exclude differences in infectivity as basis for the here observed lower virus production rate of HIV-2 and suggest that cells productively infected with HIV-2 produce less virus particles per replication cycle than cells productively infected with HIV-1. This lower virus production can be due to several viral genes that play a role at post-entry and more downstream steps in the viral replication cycle. Exact knowledge on which viral genes and steps in the viral replication cycle involved in this phenotype is currently under investigation.

Recently, Ariën et al. demonstrated that X4 HIV-2 isolates are often out-competed by X4 HIV-1 isolates in in vitro competition assays, indicating differences in replicative capacity between late stage HIV-2 and HIV-1 as well (Arien et al., 2005). Together these and our findings suggest an intrinsic difference in the replicative capacity of HIV-1 and HIV-2 in PBMC, a notion further supported by the apparently similar replication rates of HIV-1 variants from AS/LTNP and R5 HIV-2 variants from progressors. Marchant et al. recently demonstrated that HIV-1 and HIV-2 variants have different replication characteristics in macrophage cultures as well (Marchant et al., 2006).

Patients and methods

Subjects and viruses

HIV-2 biological clones were obtained from 5 long-term aviremic HIV-2-infected individuals and 6 individuals with progressive disease, all participating in the Rotterdam cohort (Table 1). With the exception of RH2-26 who is Caucasian, all HIV-2-infected individuals are of West-African origin. HIV-1 biological clones were derived from 6 asymptomatically infected individuals, four Caucasian and participants of the Amsterdam Cohort studies on HIV-1 and AIDS (ACH), two of West-African origin and participant of the Rotterdam cohort (RH). One biological HIV-1 clone isolated from an individual with AIDS was included as a reference virus. At the time of virus isolation, none of the individuals received antiretroviral therapy, with the exception of RH2-5 who was at the time treated with, but not responding to, AZT/3TC.

Biological clones were isolated by co-cultivation of patient PBMC with healthy donor PBMC in a limiting dilution fashion as described elsewhere (Blaak et al., 1998, 2004). We favor to perform virus isolation under limiting dilution conditions as this allows one to obtain virus variants with varying biological properties that co-exist within the viral quasispecies in vivo. This in contrast to bulk virus isolations where only the fittest variant(s) is (are) obtained. All virus stocks were grown simultaneously in PBMC from one healthy donor. Infectious virus titers of stocks were determined by analysis of concentrations of functional RT enzyme (Marozsan et al., 2004) using an ELISA-based RT activity detection kit (Lenti RT activity kit, Caviditech, Uppsala, Sweden). All HIV-1 and HIV-2 variants from asymptomatics were restricted to the use of CCR5 in PBMC as witnessed from their incapability to infect CCR5Δ32/Δ32 PBMC (not shown), although in GHOST cells the HIV-2 variants could use CXCR6 and GPR15 (Blaak et al., 2005) and HIV-1 variants from RH1-2 could use CXCR6 (not shown). The HIV-1 progressor variant was also restricted to the use of CCR5, the HIV-2 progressor variants could either use CCR5 or CXCR4 (Table 1).
Preparation of target cells

PBMC of healthy donors were isolated from buffy coats using Ficoll-Paque PLUS (Amersham Biosciences Europe, Freiburg Germany) and stored at −135 °C. All donors used in experiments had the CCR5+/+ phenotype. Three days prior to infection, PBMC were thawed and cultured in Iscoves modified Dulbecco’s medium (IMDM; Biowhittaker-Cambrex Bio Science, Verviers, Belgium) supplemented with 10% Fetalclone II (Hyclone, Perbio Science Nederland BV, Etten-Leur, The Netherlands), 100 U/ml penicillin, 10 μg/ml streptomycin (Biowhittaker), and 1 μg/ml phytohemagglutinin (PHA; Murex, Biotrading Benelux BV, Mijdrecht, The Netherlands). Upon stimulation, PHA-blasts were either directly used or first depleted of CD8+ T cells using anti-CD8 coated magnetic beads (Dynal Biotech GmbH, Hamburg, Germany) according to the instructions of the manufacturer. To increase depletion efficiency, two consecutive rounds of depletion were performed. After this treatment, the percentages of CD8+ T cells present in the lymphocyte population were always less than 0.1% as determined by flow cytometric analysis of cells stained with Cy5-, FITC- and PE-conjugated monoclonal antibodies directed against CD3, CD4 and CD8 respectively (DakoCytomation, Glostrup, Danmark), using standard staining procedures.

Analysis of replication kinetics

PHA-stimulated cells (1.25 or 2.0 × 10^6) resuspended in IMDM (Biowhittaker) supplemented with 10% Fetalclone II (Biowhittaker), 20 U/ml recombinant interleukin-2 (rIL-2; Proleukin, Chiron Benelux BV, Amsterdam The Netherlands) and 4 μg/ml polybrene (Pb; Sigma-Aldrich Chemie, Schnell-dorf, Germany) were exposed to inocula equalized for the concentration of functional RT enzyme as a measure for infectious virus (Maroszan et al., 2004) (250 or 500 pg RT per infection, depending on the experiment). After 1 to 1.5 h of incubation, cells were washed three times and resuspended in rIL-2/Pb-containing medium to a final concentration of 1.10^6 per ml and transferred to 24-wells plates. In the case of 2-week cultures, fresh PHA-blasts were added at day 7. Culture supernatants were sampled every 2 days starting from day 1 and stored at −70 °C. As a measure for replication, concentrations of functional RT in culture supernatants were measured (Caviditech), representing newly produced virus particles. Corrections were applied to correct for changes in culture volumes as a result of sampling and the addition of fresh cells.

Replication rates

Replication rates were calculated based on the maximal increase in RT concentration observed between the ‘lag-phase’ and the ‘plateau-phase’. Depending on virus and donor cell combination, RT accumulation rates generally started declining as of day 5 or 7, leaving only a short interval with optimal growth (generally days 3 and 5 or days 5 and 7). To be able to perform linear regression based on 2 data points, we included a forced y-intercept value of 1 (i.e., increase was set relative to ‘1’ for all variants), thereby assuming a constant replication rate as of the moment of the first burst, which is probably around day 1 (Perelson et al., 1996). Thus, obtained regression curves excellently fitted data observed during the interval of optimal growth (see Fig. 3a).

Infectivity in CD8+ T cell-depleted PBMC

CD8+ T cell-depleted cells (2 × 10^6) were inoculated, incubated for 1 h, washed and resuspended in 2 ml rIL-2/Pb-containing medium of which 1.5 ml was transferred to 24-wells plates and cultured to determine replication kinetics. Of the remaining cells, 5 4-fold dilutions were made, starting with a concentration of 4 × 10^5 cells/ml, and quadruplets of 100 μl were transferred to 96-wells plates and co-cultivated with uninfected PHA-stimulated CD8+ T cell-depleted PBMC, 1.25 × 10^5 per well. Cultures were maintained for 21 days to allow detection of virus production also in cultures where initially one infected cell was seeded. At days 7 and 14, 1/3 of the culture supernatants were removed, cultures resuspended and 1/2 of the remaining volume transferred to fresh 96-wells plates, containing freshly 3-day PHA-stimulated and CD8-depleted PBMC. At day 21, culture supernatants were harvested and analyzed for virus production. The frequency of infectious units per million exposed cells (IUPM) was estimated from the proportions of negative wells in the different cell dilutions using a maximum likelihood method (Strijbosch et al., 1987).

Single cycle infectivity in GHOST-CCR5 cells

Single cycle infectivity was determined using GHOST-CCR5 cells as described by others (Bleiber et al., 2001) with slight modifications. GHOST cells cultured in RPMI (Biowhittaker) containing 10% Fetal Bovine Serum (FBS, Biowhittaker), hygromycin (100 μg/ml), geneticin (500 μg/ml) and puromycin (1 μg/ml) were seeded in 24-well plates at 54,000 cells per well in selection medium 1 day prior to infection. Each virus (400 pg RT in 500 μl RPMI + 10%FBS + 4 μg/ml Pb) was added to 6 individual wells containing GHOST-CCR5 cells, and plates were centrifuged for 15 min at 2700 × g (spinoculation) (Pietroboni et al., 1989). Plates were incubated for another 2 h, after which inocula were removed and selection medium was reapplied. For each virus variant one well was harvested at 14, 17, 20, 23, 26 and 30 h after infection. For this purpose, culture medium was removed and cells incubated with 90 μl trypsin (GIBCO Invitrogen, Breda, The Netherlands). Trypsin was inactivated by the addition of 300 μl PBS/20% FBS and cells were kept at 4 °C until they were analyzed using a flow cytometer. Instrument settings were adjusted so that the mean fluorescence in mock-infected cells was approximately 101.

Statistical methods

Nonparametric tests were used for small groups (n < 9) and when samples were not normally distributed. In cases where log-transformation introduced normality, parametric tests were
used on log-transformed values. Normality was determined using Shapiro-Wilk. All analyses were performed using SPSS 11 for windows.

Acknowledgments

We like to thank Fransje Koning, Ronald van Rij and David Kwa for sharing their HIV-1 stocks, and Martin Schutten for subtyping the viruses isolated from the African HIV-1-infected individuals. We are grateful to all HIV-infected individuals for their participation. GHOST cells were provided by Dr. Littman and Dr. KewalRamani via the EU programme EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK (Grant number QLKZ-CT-1999-00609 and GP828102).

This study was financially supported by Zon-MW (grant number 906-02-007) and approved of by the Ethical Medical Committee (nr. 196.454/2000/221).

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