Escape from autologous humoral immunity of HIV-1 is not associated with a decrease in replicative capacity

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ABSTRACT

Autologous HIV-1-specific neutralizing antibodies (NAbs) seem unable to inhibit viral replication as they rapidly select for neutralization escape variants. However, NAbs could potentially contribute indirectly to the control of HIV-1 if changes in the viral envelope coinciding with NAb escape would impair viral replication fitness. Here we analyzed the replication kinetics of HIV-1 isolated over the course of infection from five typical progressors, three of whom developed strong autologous neutralizing humoral immunity. Viral replication rate did not correlate with viral sensitivity to autologous serum neutralization or with envelope length or number of potential N-linked glycosylation sites in gp120, suggesting that the flexibility of the viral envelope allows escape from NAbs without the loss of viral fitness. Interestingly, the appearance of rapidly replicating viruses late in infection correlated with lower CD4$^+$ T-cell counts, suggesting that this viral characteristic may be positively selected when the availability of target cells becomes limiting.

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Introduction

Upon infection with human immunodeficiency virus type 1 (HIV-1), the majority of individuals develop a potent autologous neutralizing antibody (NAb) response directed against the envelope glycoprotein (Env). The understanding of the impact of the autologous NAb response on the control of virus replication remains incomplete. Due to the rapid selection of antibody escape variants, NAbs generally lack the ability to inhibit contemporaneous viruses (Albert et al., 1990; Arendrup et al., 1992; Bunnik et al., 2008; McKeating et al., 1989; Moore et al., 2009; Richman et al., 2003; Rong et al., 2009; Von Gegerfelt et al., 1991; Watkins et al., 1993; Wei et al., 2003). The humoral immune response therefore seems ineffective in directly inhibiting virus replication. However, long-term non-progressors (LTNPs) were shown to have broader and more potent NAb responses than progressors (Cao et al., 1995; Cecilia et al., 1999; Zhang et al., 1997), although other studies did not confirm this finding (Bailey et al., 2006; Harrer et al., 1996). Moreover, the development of anti-simian immunodeficiency virus (SIV) humoral immunity was found to be critical for the control of SIV infection in a macaque model (Miller et al., 2007). These observations suggest that, although NAbs may not be able to directly inhibit replication of HIV-1, disease progression may be influenced by the development of a potent autologous NAb response.

Mechanisms by which HIV-1 can obtain resistance to antibody neutralization include extensions of the variable loops of Env (Bunnik et al., 2008; Sagar et al., 2006), possibly to protect underlying, conserved regions from antibody recognition (Gray et al., 2007), and an increase in the density of the envelope glycan shield (Bunnik et al., 2008; Sagar et al., 2006; Wei et al., 2003). These large alterations in the length of the variable loops and the glycosylation level of Env are indicative of the strong selective pressure exerted on the protein by NAbs. We have previously observed that changes in the length of the variable loops and the level of glycosylation revert in later stages of infection (Bunnik et al., 2008), when selection pressure may be lost due to an impairment of host immunity. Moreover, NAb-driven changes in Env (partially) revert upon transmission, which renders the recipient virus neutralization sensitive, although this may not be true for all subtypes of HIV-1 and/or all modes of transmission (Chohan et al., 2005; Derdeyn et al., 2004; Li et al., 2006; Sagar et al., 2009). Similarly, reversion of CTL escape mutations also occurs upon transmission of HIV-1 to an HLA class I-mismatched recipient, the rate of which is associated with the cost of the specific mutation to viral fitness (Li et al., 2007; Loh et al., 2007). The observation that HIV-1 Env reduces its epitope-masking mechanisms in the absence of humoral immune pressure may thus indicate that escape from NAbs influences the replicative fitness of HIV-1. The development of a potent autologous NAb response may in this way contribute to indirect control of virus replication.

We previously reported on the changes in length and glycosylation of gp120 that coincided with escape from autologous neutralizing immunity in a longitudinal study on five participants of the Amsterdam Cohort Studies with varying humoral immune responses.

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In our present study, we analyzed the effect of escape from autologous humoral immunity on the replication kinetics of HIV-1 and correlated the observed changes in length and glycosylation of gp120 of viruses isolated from these five individuals with the viral replication rate, both early in infection, when escape from NAbs occurred, as well as late in infection, when reversion of the changes induced by NAbs escape was observed. In addition, we investigated the influence of other selective processes on the replication rate of late-stage HIV-1.

Results

Replication kinetics of HIV-1 over the course of infection

We investigated whether changes in Env that related to escape from autologous neutralizing humoral immunity influenced the replication kinetics of HIV-1, using virus variants that were isolated at multiple time points during infection from five typical progressors of the ACS. In a previous study, we determined that individuals H1, H2, and H3 developed high-titer autologous neutralizing activity, which decreased in potency in the chronic phase of infection (Bunnik et al., 2008). Patients H4 and H5, in whom autologous neutralizing serum activity was not detected, were also included in this study to be able to distinguish between the effect of escape from NAbs and the effect of other selective processes on viral replication rates. All patients received antiretroviral (mono)therapy for various periods of time over the course of infection (Bunnik et al., 2008), selecting for drug resistance mutations in *pol* that have been described to reduce viral replication fitness (Harrigan et al., 1998; Harrigan et al., 2000) (data not shown). To exclude an effect of these drug resistance mutations and of additional mutations in other genes than *env* on the viral

replication rate, we generated a panel of chimeric NL4-3 viruses, in which the original envelope was replaced with the envelopes of virus variants that were isolated from our five patients. For each time point, envelopes from a minimum of 2 and a maximum of 8 viruses were analyzed.

We determined the in vitro replicative capacity of HIV-1 over the course of infection expressed as the cumulative p24 production at day 4 after inoculation of PHA-stimulated PBMCs with the various chimeric NL4-3/Env variants. In all patients, the average replication rates of HIV-1 variants present at a certain time point changed over the course of infection, although different patterns of change were observed. For patients H1 and H4, viruses that had been isolated during the asymptomatic phase of infection, at 42 months and 93 months after seroconversion, respectively, had increased replication kinetics compared to viruses that had been isolated relatively early in infection (Fig. 1A). In contrast, replication rates of viruses from patient H3 declined in this phase of asymptomatic infection, while replication kinetics of viruses from patients H2 and H5 remained the same. For viruses from patient H1, the increase in replication kinetics early in infection was followed by a decrease in replication rate in the later stages of disease, which was not observed for patient H4 (Fig. 1A). Finally, for patients H2, H3, and H5, the replication rate of late-stage virus variants was increased compared to viruses isolated at earlier time points (Fig. 1A).

Escape from autologous humoral immunity is not associated with a decrease in viral replicative capacity

Previously, we determined that patients H1, H2, and H3 developed strong autologous neutralizing activity, which decreased in potency
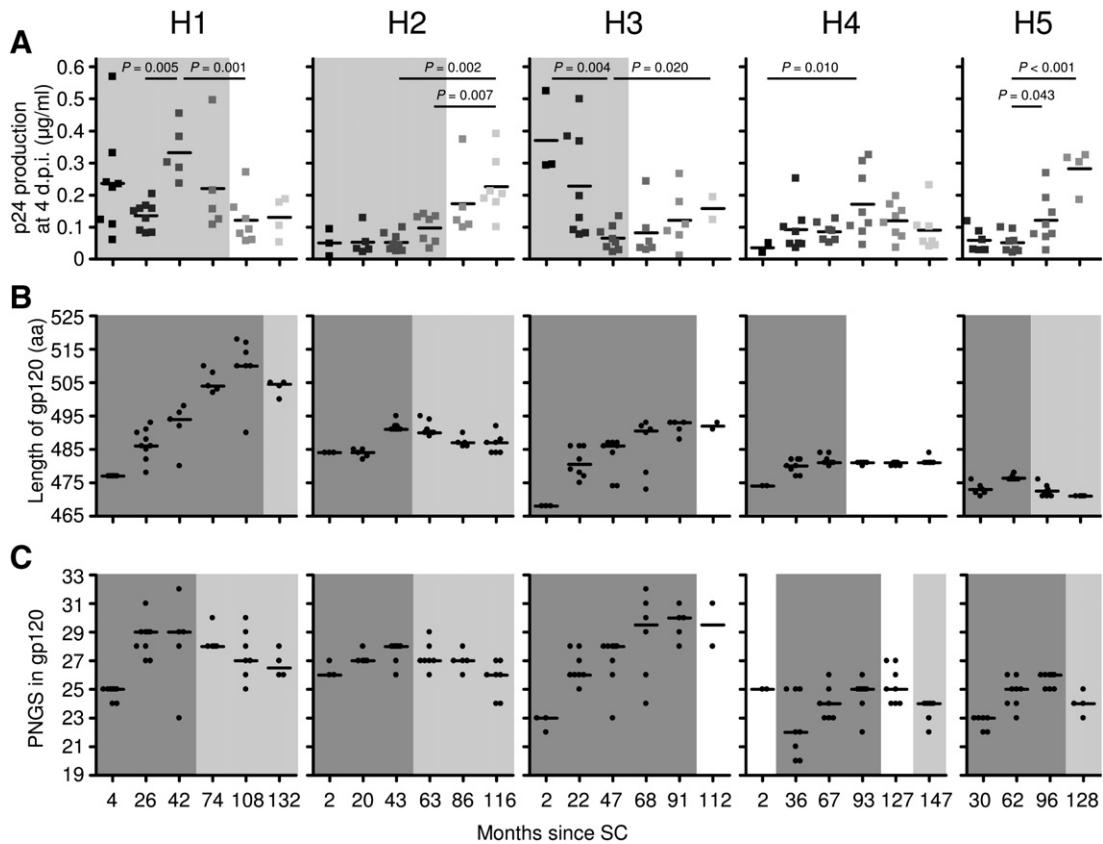

Fig. 1. Replication kinetics and molecular characteristics of gp120 of viruses isolated over the course of infection from five typical progressors. (A) Replication rates of individual chimeric NL4-3/Env variants expressed as the cumulative p24 production at day 4 after infection of PHA-stimulated PBMC. The time points at which viruses were likely to be subject to NAb pressure are indicated in grey. Data were analyzed using a *t* test for independent samples. (B) The length of gp120 and (C) the number of PNGS in gp120 of clonal HIV-1 variants over the course of infection. Increases and decreases in the length of number of PNGS over time are indicated by dark grey and light grey areas, respectively. In all panels, the horizontal bars represent the means. D.p.i, days post-infection; aa, amino acids; PNGS, potential N-linked glycosylation sites; SC, seroconversion.

during chronic infection (Bunnik et al., 2008). Virus variants from these patients were not neutralized by sera from the same time point or from earlier time points, suggestive of viral escape from neutralizing humoral immunity. The time points at which viruses were likely to be subject to NAb pressure (i.e., time points when viruses were sensitive to autologous serum neutralization and/or had escaped from the neutralizing activity elicited in response to virus variants from the previous time point) are indicated by grey-shaded areas in Fig. 1A. We first analyzed whether escape from the autologous NAb response was accompanied by a decrease in replicative fitness. Fig. 1A shows that the replicative capacity of viruses from patient H3 decreased during the first three time points, coinciding with the presence of autologous neutralizing activity. However, for patient H3 as well as for patients H1 and H2, the sensitivities of individual clonal HIV-1 variants for autologous serum from both the contemporaneous and the subsequent time point (expressed as 50% inhibitory concentrations) did not correlate with the replication rates of the corresponding chimeric NL4-3/Env variants (data not shown).

Escape from autologous neutralizing humoral immunity coincided with an increase in the length of the variable loops and the number of PNGS in envelope (Figs. 1B and C) (Bunnik et al., 2008), although differences in neutralization sensitivity between viruses from the same time point could not be explained by variations in the envelope length or the number of PNGS. Moreover, changes in the length of the variable loops and the number of PNGS in Env were also observed in viruses from patients H4 and H5, who did not develop a detectable autologous NAb response, albeit that both masking mechanisms remained at a lower level in viruses from these patients as compared to viruses from patients H1, H2, and H3 (Figs. 1B and C). To study whether the increased protection of the viral envelope against autologous humoral immunity is associated with a reduction in the replicative capacity of HIV-1, we correlated the length and the number of PNGS in gp120 from virus variants that were isolated in the early phase of infection of patients H1, H2, and H3, when humoral immunity could be detected, to the replication rates of the corresponding chimeric NL4-3/Env variants. In patients H1 and H2, length and glycosylation characteristics of gp120 were not associated with the viral replication rate (Figs. 2A and B). In contrast, a significant negative correlation was observed between both the length of gp120

and the number of PNGS in gp120 of viruses isolated during the first part of infection from patient H3, and the replication kinetics of the corresponding chimeric NL4-3/Env variants (Figs. 2A and B). However, it should be mentioned that the chimeric NL4-3 variants expressing Env from viruses isolated at the earliest time point from patient H3 replicated exceptionally rapid compared to NL4-3 variants expressing early Envs from the other patients. The subsequent decline in replication rate to relatively normal levels, which coincided with increases in gp120 length and the number of PNGS in this gene, may thus have been driven by other selective processes than escape from NAbs. Of note, increases in length and glycosylation characteristics of Env also occurred in viruses of patients H4 and H5, albeit to lower levels, and were for HIV-1 variants from these patients also not correlated with replication rate (data not shown).

Increased viral replication kinetics in late-stage disease

In most patients, changes in Env that coincided with escape from NAb pressure early in infection reverted during later disease stages (Figs. 1B and C) (Bunnik et al., 2008). To investigate whether this reversion of envelope masking mechanisms allowed higher levels of replication, we correlated the envelope length and glycosylation characteristics of viruses that had been isolated at four time points late in infection to the replication rate of the corresponding chimeric NL4-3/Env variants. In patients H1 and H4, replication rates of late chimeric NL4-3/Env variants were similar compared to chimeric NL4-3 variants expressing Envs isolated from earlier time points and, as a consequence, did not correlate with the envelope length or the number of PNGS in gp120 (data not shown). In contrast, the replication rates of chimeric NL4-3 variants expressing Envs from viruses isolated late in infection from patients H2, H3, and H5 showed increased replication kinetics compared to earlier NL4-3/Env variants. In patients H2 and H5, this increased replication rate of late-stage virus variants was negatively correlated with both the length and the number of PNGS in gp120 (Figs. 3A and B).

Increased replication of late-stage viruses coincides with low CD4 counts

Previous studies have shown that the replication fitness of R5 viruses isolated during late-stage disease is increased (Etemad et al.,
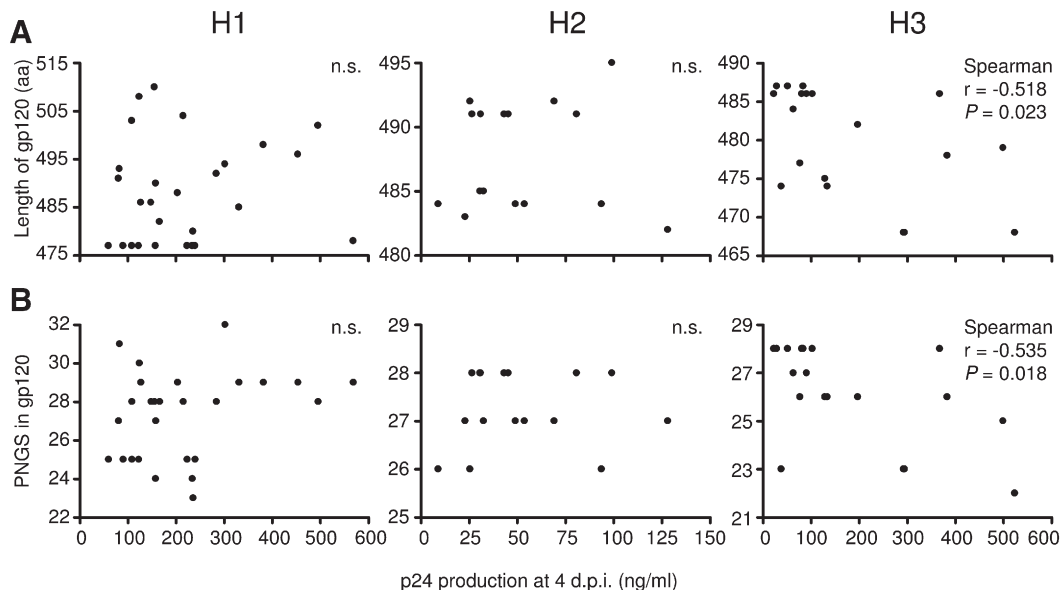

Fig. 2. Replication kinetics of HIV-1 upon escape from the autologous NAb response. Correlation between the cumulative production of p24 at day 4 after infection of chimeric NL4-3 variants expressing Env of viruses isolated from patients who developed strong autologous humoral immunity, and the length of gp120 (A) or the number of PNGS in gp120 (B). Correlations were evaluated using the Spearman's rank test. Aa, amino acids; PNGS, potential N-linked glycosylation sites; d.p.i, days post-infection.
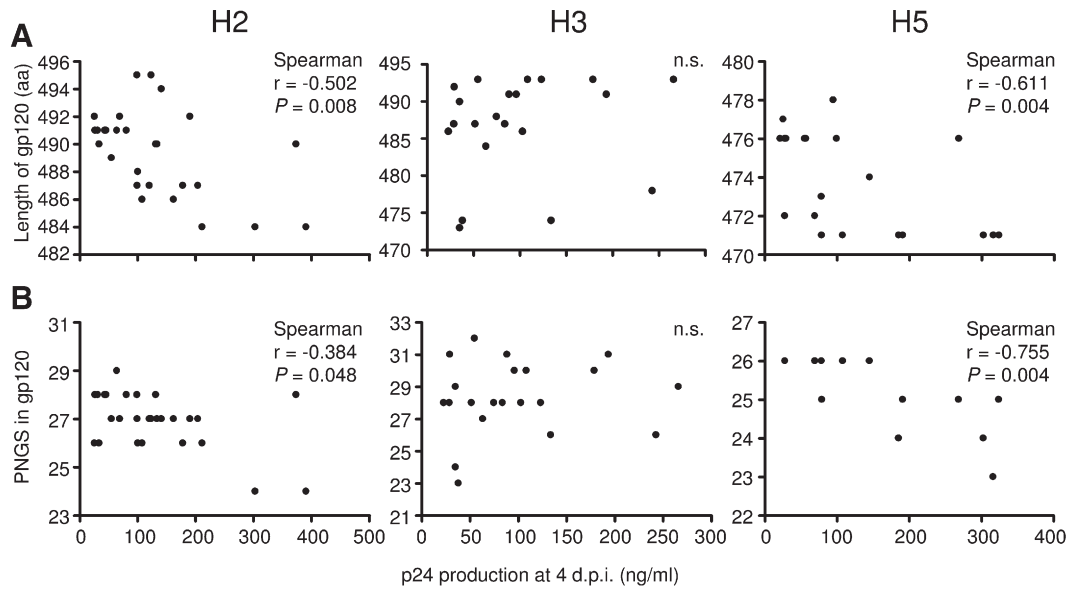
Fig. 3. Replication kinetics of HIV-1 in relation to molecular characteristics of gp120 in during chronic infection. Correlation between the cumulative production of p24 at day 4 after infection of chimeric NL4-3 variants expressing Env from viruses isolated during the last four time points of infection, and the length of gp120 (A) or the number of PNGS in gp120 (B). Correlations were analyzed using the Spearman's rank test. Aa, amino acids; PNGS, potential N-linked glycosylation sites; d.p.i., days post-infection.

2009; Repits et al., 2005; Repits et al., 2008; Sterjovski et al., 2007). Here, we could confirm this observation for viruses from patients H2, H3, and H5, while chimeric NL4-3 variants expressing Env from viruses isolated during the later disease stages from patients H1 and H4 did not exhibit enhanced replication rates. To understand this observation and to examine whether other factors than decreased envelope masking mechanisms may explain the enhanced replication rate of late-stage viruses from patients H2, H3, and H5, we plotted the CD4$^+$ T-cell counts of all patients over their course of infection. In patients H1 and H4, the CD4$^+$ T-cell counts remained above 100 cells/μl blood, while the appearance of rapidly replicating virus variants in patients H2, H3, and H5 coincided with a CD4$^+$ T-cell count below 100 cells/μl blood (Fig. 4). Although this threshold of 100 CD4$^+$ T cells/μl blood was chosen arbitrarily, these results indicate that the increased replication kinetics of HIV-1 may result from the adaptation of the virus to replicate in the presence of extremely low numbers of target cells.

Discussion

The impact of the autologous NAb response on HIV-1 disease progression remains incompletely understood. As HIV-1 rapidly escapes from autologous humoral immunity, NAbs are not able to directly inhibit the spread of the virus. However, if escape from NAbs would result in a decrease in viral fitness, analogous to what is

observed for certain CTL escape mutations (Friedrich et al., 2004; Leslie et al., 2004), strong autologous humoral immunity could contribute indirectly to the control of virus replication. In this study, we investigated whether escape of HIV-1 from the autologous NAb response coincided with changes in viral replication kinetics.

In general, the replication rates mediated by Envs of HIV-1 variants isolated from individuals who developed strong autologous humoral immunity did not correlate with the sensitivities of these viruses for autologous serum or with molecular characteristics of Env that have been associated with NAb escape. Although it should be mentioned that these results were obtained using a relatively small study cohort, the absent correlation between escape from NAbs and viral replication fitness may suggest that the development of a potent NAb response does not result in the control of viral replication. Indeed, the plasma viral loads in patients H1, H2, and H3, who all developed an autologous NAb response, were not lower as compared to patients H4 and H5, in whom neutralizing activity in serum was not detected (Bunnik et al., 2008). These observations are in agreement with recent studies showing that the development of a broad NAb response does not influence disease progression (Euler et al., in press; Piantadosi et al., 2009). The previously observed presence of strong humoral immunity in LTNPs is therefore more likely a consequence of preserved immune surveillance rather than a cause of non-progression.

Our current data show that the replication kinetics of HIV-1 do not change upon escape from the autologous NAb responses. This may
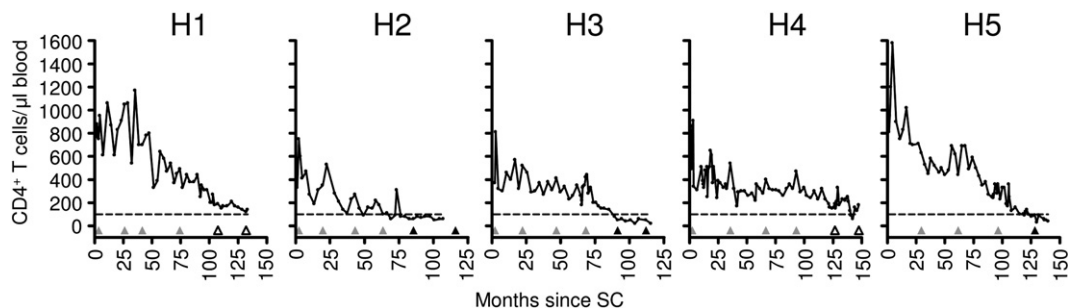

Fig. 4. CD4$^+$ T-cell counts over the course of infection. The horizontal dashed line is drawn at 100 CD4$^+$ T cells/μl blood. Time points of virus isolation are depicted by triangles. Open triangles indicate time points during symptomatic disease at which time CD4$^+$ T-cell counts are above 100 (patients H1 and H4), while closed triangles represent time points during symptomatic disease at which time CD4$^+$ T-cell counts are below 100 (patients H2, H3, and H5).

suggest that autologous neutralizing activity is mainly directed against the variable loops in envelope in which a high degree of sequence variation may be allowed without impairment of the viral replication fitness. In addition, we have previously shown that HIV-1 resistance to broadly neutralizing monoclonal antibodies b12, 2G12, 2F5, and 4E10 did also not coincide with lower replication rates as compared to the replication kinetics of neutralization sensitive viruses (Quakkelaar et al., 2007a). These findings suggest that the flexibility of the viral envelope allows escape from NAbs by the introduction of changes in the sequence or conformation of both its variable and conserved regions, possibly in combination with compensatory mutations, without the loss of viral fitness.

Early in infection in patient H3, and late in infection in patients H2 and H5, the length and number of PNGS in gp120 correlated with the viral replication rate, indicating that the replication kinetics of HIV-1 may under certain circumstances be facilitated by shorter variable loops and/or a lower numbers of glycans on the viral envelope. However, the observed changes in replication rate may also have been selected by other mechanisms, resulting in a temporal but not a causal relationship between HIV-1 replication capacity and molecular characteristics of Env. Indeed, the increasing viral replication rate of late-stage viruses in patients H2, H3, and H5 seemed to correlate stronger with a CD4$^+$ T-cell count below 100 cells/μl blood than with the length or the number of PNGS in gp120. It has previously been shown by us (Koning et al., 2003) and others (Etemad et al., 2009; Gray et al., 2005; Jansson et al., 1996; Jansson et al., 1999; Karlsson et al., 2004; Repits et al., 2008; Sterjovski et al., 2007) that late-stage R5 viruses evolve towards a more efficient usage of CCR5. The adaptation of HIV-1 to persist in the presence of low levels of CD4$^+$ T cells may thus result in an increased replication capacity (Etemad et al., 2009; Repits et al., 2008), although we cannot exclude that the enhanced loss of CD4$^+$ T cells is a consequence of the increased viral replication rate late in infection.

The selective outgrowth of virus variants with a reduced number of PNGS and shorter variable loops in late-stage disease (Bunnik et al., 2008) may suggest that virus replication is hindered by long variable loops and/or an excess of glycosylation of the viral envelope when these features are not required to protect against neutralizing antibodies. Although our results do not support this hypothesis, previous studies have shown that minor variations in cell growth in the parallel cell cultures of an in vitro replication assay may prevent the detection of relatively small differences in replication rates (van Opijnen et al., 2004). A technique better suitable for the detection of small differences in viral fitness is a head-to-head competition assay (van Opijnen and Berkhout, 2005). However, these competition assays are extremely labour-intensive, especially for the large number of viruses that were tested here ($n = 172$). Thus, based on our results, it cannot be excluded that escape from NAbs exerts minor fitness costs on HIV. However, as evidence is accumulating that the development of a broad NAb response does not protect from disease progression (Euler et al., in press; Piantadosi et al., 2009), it seems unlikely that such small fitness costs are relevant for HIV-1 pathogenesis.

In summary, we have shown that escape from autologous humoral immunity generally does not impair the replication kinetics of HIV-1 and will therefore not result in control of virus replication. Viral replication kinetics may, however, be influenced by other selective processes, such as the adaptation to a low availability of target cells in late-stage disease.

Materials and methods

Patient and viruses

The patients in our present study were homosexual male participants of the Amsterdam Cohort Studies on HIV infection and AIDS (ACS) who seroconverted during active follow-up and who

progressed to AIDS within 7–11 years after infection in the presence of subtype B CCR5-using (R5) HIV-1 variants only, as shown by absent virus replication in 3-monthly performed cocultures of patient peripheral blood mononuclear cells (PBMCs) and the MT2 cell line. For better readability, patient identifiers were recoded as H1 (ACH19999), H2 (ACH19542), H3 (ACH18969), H4 (ACH19768), and H5 (ACH19659), which correspond to the identifiers used in a previous study (Bunnik et al., 2008). Previously, we determined that patients H1, H2, and H3 developed high-titer autologous neutralizing activity, whereas autologous neutralizing activity was not detected in serum from H4 and H5 (Bunnik et al., 2008). From these individuals, we previously isolated clonal HIV-1 variants from PBMCs (Schuitemaker et al., 1992; Van 't Wout et al., 2008) that were obtained at six time points covering the disease course from SC up to 2 to 3 years after clinical AIDS diagnosis (Bunnik et al., 2008). For patient H5, attempts to isolate clonal HIV-1 variants from PBMCs obtained at SC and at time points after AIDS diagnosis were not successful. For further study, we selected a maximum of eight virus variants per patient per time point, representing the variation in time observed between the start of the clonal virus isolation procedure and the first day of detectable p24 production in the microculture for viruses of that time point.

Cells

Experiments were performed using cryopreserved pooled PBMCs isolated from buffy coats obtained from 12 healthy seronegative blood donors by Ficoll-Isopaque density gradient centrifugation. Cells were thawed and stimulated for 3 days in IMDM (Lonza) supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml; Invitrogen), ciproxin (5 μg/ml; Bayer), and phytohemagglutinin (PHA, 5 μg/ml; Oxoid) at a concentration of 5×10^6 cells/ml. Subsequently, PBMCs (10^6/ml) were grown in the absence of PHA, in medium supplemented with recombinant interleukin-2 (20 U/ml; Chiron Benelux) and polybrene (5 μg/ml, hexadimethrine bromide; Sigma).

Sequence analysis

Envs of clonal HIV-1 variants were amplified from DNA that was isolated from in vitro infected healthy donor PBMCs. *Env* PCR products were subsequently sequenced as described previously (Beaumont et al., 2001; Boom et al., 1991; Quakkelaar et al., 2007b). Nucleotide sequences of all virus clones per individual were aligned using ClustalW in the software package of BioEdit (Hall, 1999) and edited manually. Potential N-linked glycosylation sites were identified using N-GlycoSite (Zhang et al., 2004) at the HIV database Web site (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). Coreceptor usage was predicted by PSSM based on the V3 sequences (Brumme et al., 2004; Jensen et al., 2003), using Web PSSM at http://indra.mullins.microbiol.washington.edu/pssm/.

Preparation of chimeric viruses

Env fragments from HXB2 nucleotides (nt) 5658 to 9171 were amplified by PCR using Expand High Fidelity PCR System (Roche Applied Science). Chimeric NL4-3/Env viruses were produced by homologous recombination of the *env* PCR products with a pNL4-3 vector (a kind gift from P. Alcami). In short, pNL4-3 was restricted with *Xba*I (HXB2 nt 6114) and *Xho*I (HXB2 nt 8898) and was subsequently cotransfected with an *env* PCR product into 293T cells in a 24-well plate using the calcium phosphate method. After 2 days, PHA-stimulated PBMCs from healthy seronegative blood donors were added to the culture, and the next day, the PBMCs were transferred to a culture flask. Supernatants were harvested when positive for p24, as

determined using an in-house p24 antigen capture enzyme-linked immunosorbent assay (Tersmette et al., 1989). The presence of the correct *env* in NL4-3 was confirmed by sequencing.

Characterization of HIV-1 replication kinetics

PHA-stimulated healthy donor PBMCs (2×10^6) were inoculated with 500 TCID$_{50}$ of a given chimeric NL4-3/Env virus in a total volume of 2 ml for 2 h at 37 °C in a shaking water bath. Subsequently, cells were washed with 10 ml of IMDM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and resuspended at a concentration of 10^6 cells/ml for culture. Fresh PHA-stimulated PBMCs (10^6) in a volume of 1 ml were added at day 5 and day 8. Cultures were maintained for 11 days. Samples (75 μl) for the determination of p24 antigen production in culture supernatant were harvested each day. The concentration of p24 in all samples was determined at the same time using an in-house p24 antigen capture ELISA and was used to calculate the p24 production per milliliter of supernatant by correcting for the differences in the volumes of culture supernatant.

Statistical analysis

Statistical analyses were performed in SPSS 16 software package. Differences in replication rates were assessed using a t test for independent samples. Correlations between replication kinetics and the length or number of PNGS in Env were evaluated using the Spearman's rank test.

Acknowledgments

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