

Emergence of monoclonal antibody b12-resistant human immunodeficiency virus type 1 variants during natural infection in the absence of humoral or cellular immune pressure

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Human immunodeficiency virus type 1 (HIV-1) resistance to broadly neutralizing antibodies such as b12, which targets the highly conserved CD4-binding site, raises a significant hurdle for the development of a neutralizing antibody-based vaccine. Here, 15 individuals were studied of whom seven developed b12-resistant viruses late in infection. The study investigated whether immune pressure may be involved in the selection of these viruses *in vivo*. Although four out of seven patients showed HIV-1-specific broadly neutralizing activity in serum, none of these patients had CD4-binding site-directed antibodies, indicating that strong humoral immunity is not a prerequisite for the outgrowth of b12-resistant viruses. In virus variants from one patient, who showed extremely weak heterologous and autologous neutralizing activity in serum, mutations were identified in the envelope that coincided with changes in b12 neutralization sensitivity. Lack of cytotoxic T-cell activity against epitopes with and without these mutations excluded a role for host cellular immunity in the selection of b12-resistant mutant viruses in this patient. However, b12 resistance correlated well with increased virus replication kinetics, indicating that selection for enhanced infectivity, possibly driven by the low availability of target cells in the later stages of disease, may coincide with increased resistance to CD4-binding site-directed agents, such as b12. These results showed that b12-resistant HIV-1 variants can emerge during the course of natural infection in the absence of both humoral and cellular immune pressure, suggestive of other mechanisms playing a role in the selective outgrowth of b12-resistant viruses.

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INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) is highly adapted to impede recognition of its conserved regions by the humoral immune response (Chen *et al.*, 2005; Decker *et al.*, 2005; Edwards *et al.*, 2001; Kwong *et al.*, 1998; Wyatt *et al.*, 1998). Nevertheless, neutralizing antibodies directed against such

conserved epitopes are considered to be an essential component of a preventative vaccine against HIV-1 (Burton, 2002; Burton *et al.*, 2004; Desrosiers, 2004). Although attempts to induce a potent and broadly neutralizing antibody response have thus far been unsuccessful, a small number of broadly neutralizing monoclonal antibodies (mAbs) have been identified (Burton *et al.*, 1994; Muster *et al.*, 1993; Stiegler *et al.*, 2001; Trkola *et al.*, 1995), suggesting that the elicitation of antibodies of similar specificity and breadth by vaccination should be possible.

A supplementary figure showing sensitivity for neutralization by mAbs 2G12, 2F5 and 4E10 of longitudinally obtained virus variants is available with the online version of this paper.

One of the most promising leads in vaccine development is mAb b12, which binds to a conformational epitope on the gp120 subunit that has a distinct overlap with the conserved CD4-binding site (Kwong *et al.*, 1998; Saphire *et al.*, 2001; Zhou *et al.*, 2007). Binding of the heavy chain of b12 to the surface of gp120 blocks attachment of CD4 and thus prevents the entry of HIV-1 into a target cell (Saphire *et al.*, 2001; Zhou *et al.*, 2007). The interaction between b12 and gp120 is centred around the CD4-binding loop spanning residues 364–373, but involves many other residues in gp120 (Pantophlet *et al.*, 2003; Zhou *et al.*, 2007).

Although b12 neutralizes a broad range of primary HIV-1 variants, a substantial proportion of viruses of both B and non-B subtypes is resistant to neutralization by b12 (Binley *et al.*, 2004; Burton *et al.*, 1994). HIV-1 may obtain resistance to b12 neutralization by substitutions at b12 contact residues resulting in disruption of the b12 epitope or by changes in the conformation of Env that limit the accessibility of b12 to the epitope (Wu *et al.*, 2009). These b12-resistant viruses may be selected due to escape from b12 neutralization, as has been shown to occur both *in vitro* (Mo *et al.*, 1997) and *in vivo* (Poignard *et al.*, 1999) in the presence of relatively high concentrations of b12 ($>10 \mu\text{g ml}^{-1}$). However, we have shown recently that b12-resistant virus variants emerge during natural infection in the absence of autologous neutralizing serum activity (Bunnik *et al.*, 2009), indicating that escape from antibody neutralization may not be the only selection pressure that favours outgrowth of b12 neutralization-resistant viruses.

In this study, we identified a total of seven participants of the Amsterdam Cohort Studies on HIV infection and AIDS (ACS) (out of a group of 15 individuals who were analysed) in whom virus variants isolated late in infection were more resistant to neutralization by b12 than early viruses. Serum of these patients did not show evidence of CD4-binding site-directed neutralizing activity. Moreover, the breadth of neutralizing serum activity in these patients was not a prerequisite for the emergence of b12-resistant viruses late in infection. Virus variants of one individual, who showed extremely weak heterologous neutralizing activity in serum, were studied in more detail to understand better how b12-resistant viruses may be selected *in vivo* in the absence of antibody pressure.

RESULTS

Increasing resistance to b12 neutralization of primary HIV-1 obtained during natural infection

In a previous study, we observed an increase in resistance to b12 neutralization in virus variants isolated late in infection in three out of five individuals (patients H3, H4 and H5, see Methods for numbering; Bunnik *et al.*, 2009). To study whether an increased resistance to b12-mediated neutralization is a relatively common phenomenon for HIV-1 variants in the later stages of infection, longitudinally isolated clonal HIV-1 variants from ten additional partici-

pants of the ACS were analysed for their sensitivity to b12 neutralization using a peripheral blood mononuclear cell (PBMC)-based assay. In seven individuals out of the total of 15 individuals analysed (47%), virus variants isolated early in infection were sensitive to b12 neutralization, whilst b12 neutralization-resistant viruses emerged later in infection (Fig. 1, and data not shown). A decreasing neutralization sensitivity during the course of infection was not observed for the broadly neutralizing antibodies 2G12, 2F5 (with the exception of patients H7 and H9) and 4E10, suggesting that this change was specific for b12 (Bunnik *et al.*, 2009, and Supplementary Fig. S1, available in JGV Online).

Broadly neutralizing activity in autologous serum is not a prerequisite for the outgrowth of b12-resistant viruses

The emergence of b12-resistant virus variants late in infection may be the result of escape of these viruses from potentially neutralizing autologous antibodies directed against the CD4-binding site. As neutralizing antibodies targeting a conserved region such as the CD4-binding site may be expected to exert neutralizing activity against a variety of HIV-1 variants, we analysed the neutralizing activity of sera obtained from these seven patients approximately 4 years after seroconversion (SC) against 23 heterologous HIV-1 variants pseudotyped with envelopes from subtypes A, B, C or D using a U87 target cell-based assay (Euler *et al.*, 2010; Schweighardt *et al.*, 2007). For this tier two–three virus panel consisting of five to seven moderately neutralization-sensitive and relatively neutralization-resistant variants per subtype, broadly neutralizing serum activity was defined as neutralization of $\geq 50\%$ of viruses per subtype at serum dilutions higher than 1:100 for at least three of the four subtypes tested (van Gils *et al.*, 2009). The neutralizing activity of the sera from our patients against individual viruses from this panel has been reported previously (van Gils *et al.*, 2009). Overall, sera from four out of seven patients (H3, H6, H8 and H9) exhibited broadly neutralizing activity, whilst sera from patients H4, H5 and H7 did not (Table 1). In particular, neutralizing activity in serum from patient H5 was extraordinarily weak, neutralizing none of the heterologous virus variants with a 50% inhibitory concentration (IC_{50}) >100 (Table 1). We subsequently analysed the neutralizing activity in the serum of our patients against wild-type HIV-1 strain LAI, which is sensitive to neutralization by b12 and soluble CD4 (sCD4; $\text{IC}_{50}=0.95$ and $1.14 \mu\text{g ml}^{-1}$, respectively), and against a LAI variant with mutations K178T/Q389P, which showed a >15 -fold increase in neutralization resistance to both b12 and sCD4 ($\text{IC}_{50}=14.28$ and $>25 \mu\text{g ml}^{-1}$, respectively), but not to non-CD4-binding site-directed agents (Fig. 2). The generation of this mutant LAI variant is described below. Serum from our seven patients did not show a difference in neutralizing activity against wild-type LAI and mutant LAI, indicative of the absence of CD4-binding site-directed antibodies (Fig. 2). These results indicated that b12-resistant virus

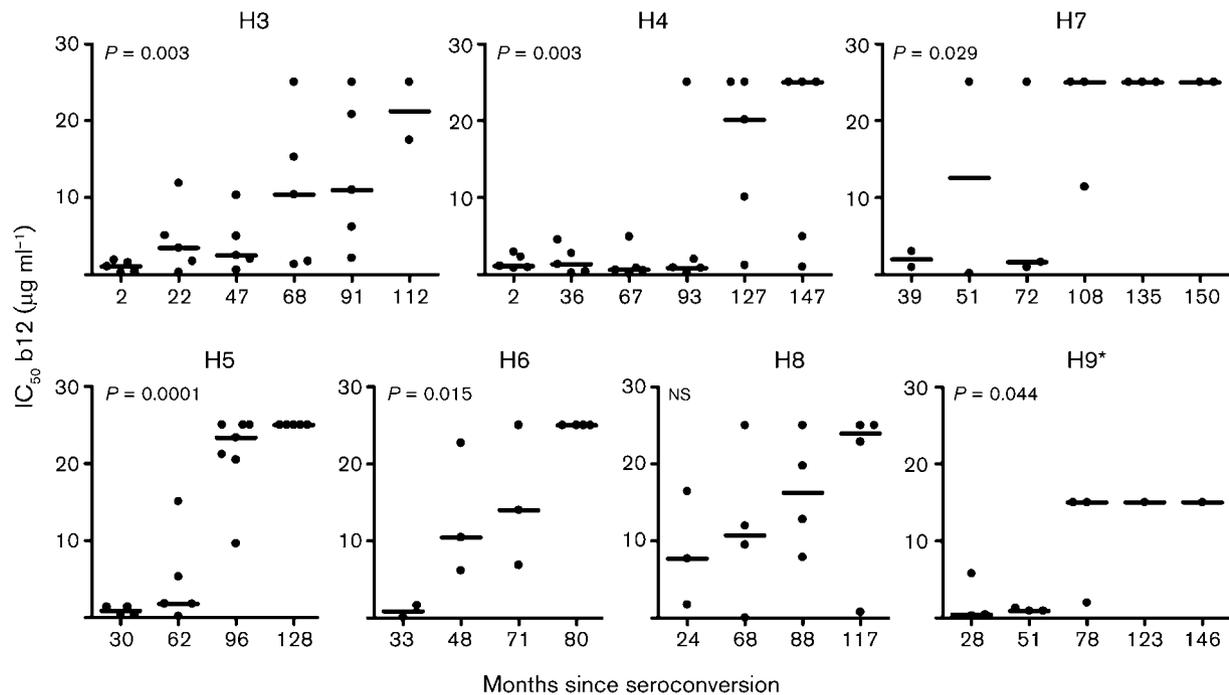


Fig. 1. Sensitivity to neutralization by mAb b12 of longitudinally obtained virus variants from seven participants of the ACS. IC_{50} values of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC_{50} values. Changes in neutralization sensitivity of viruses from the first two time points compared with virus variants from the last two time points were assessed using a Mann–Whitney U test. *, The maximum concentration of b12 used to test viruses from patient H9 was $15 \mu\text{g ml}^{-1}$. IC_{50} , 50% inhibitory concentration; NS, not significant.

variants can emerge in the absence of strong heterologous neutralizing activity and/or in the absence of neutralizing activity targeting the CD4-binding site, which suggests that other processes may be involved in their selection *in vivo*.

b12-resistant viruses from patient H5 have increased replication kinetics

The serum of patient H5 lacked cross-reactive neutralizing activity, as shown here, as well as autologous neutralizing

activity, as shown previously (Bunnik *et al.*, 2008). Moreover, b12-sensitive viruses were rapidly replaced by b12-resistant viruses in this patient, without a period in which sensitive and resistant viruses co-existed or in which viruses with intermediate neutralization sensitivity were present, as was the case in the other patients. Therefore, we decided to study the appearance of b12-resistant virus variants in patient H5 in more detail. First, we determined the replicative capacity of the b12-sensitive virus variants that were present during the early asymptomatic phase of

Table 1. Serum neutralization capacity against a panel of 23 heterologous virus variants from different subtypes, expressed as the percentage of virus variants neutralized per subtype

Heterologous neutralizing serum activity in serum samples was obtained approximately 4 years after SC from seven participants of the ACS in whom virus variants that were resistant to b12 neutralization emerged at later stages of infection. For this panel, broadly neutralizing serum activity is defined as neutralization of at least 50% of the viruses per subtype with IC_{50} values at a reciprocal serum dilution of >100 (as indicated in bold) for at least three of the four subtypes tested.

HIV-1 subtype	n	Patient (months since SC)						
		H3 (48.5)	H4 (48.1)	H5 (49.6)	H6 (48.0)	H7 (47.5)	H8 (58.9)	H9 (54.7)
A	5	80	60	0	100	0	60	60
B	6	67	17	0	100	33	67	100
C	7	57	57	0	86	14	57	100
D	5	40	20	0	80	0	40	100

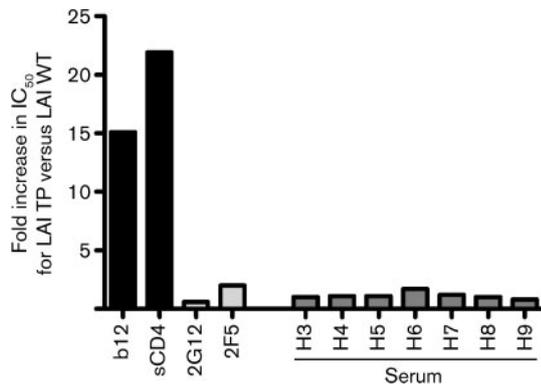


Fig. 2. Neutralizing serum activity against neutralization-sensitive wild-type (WT) LAI and the LAI mutant K178T/Q389P (TP), which is resistant to neutralization by CD4 and b12, but not to neutralization by the non-CD4-binding site-directed antibodies 2G12 and 2F5. Results are expressed as fold increase in IC_{50} for LAI TP compared with LAI WT.

infection and of the b12-resistant viruses that had emerged later in infection. To exclude an effect of potential mutations in genes other than *env* on the virus replication rate, we generated a panel of chimeric strain NL4-3 viruses, in which the original envelope was replaced with the envelopes of virus variants that were isolated from patient H5. For each time point, envelopes from a minimum of four and a maximum of seven viruses were analysed. Chimeric viruses expressing envelopes from variants isolated late in infection (95 and 128 months after SC, respectively) replicated faster compared with chimeric viruses containing envelopes derived from viruses that were isolated early in infection (30 and 62 months after SC, respectively; Fig. 3a). Moreover, the higher replication rate was significantly correlated with resistance to b12 neutralization of the original clonal HIV-1 variants isolated from patient H5 (Fig. 3b; Spearman $r=0.691$, $P=0.001$).

Identification of mutations in gp120 of viruses from patient H5 that coincide with increased resistance to b12 neutralization

To identify amino acid residues in the viral Env protein that may play a role in the increased resistance to b12 neutralization and the increased replication kinetics observed for chimeric viruses expressing Env from viruses from patient H5, gp120 sequences from variants obtained from the earliest two time points were compared with those from viruses isolated from the last two time points. At six positions in gp120, amino acid substitutions were observed in the b12-resistant, rapidly replicating virus variants that were completely absent in the b12-sensitive, slowly replicating viruses: I154M in variable region 1 (V1), K178T in V2, Q389P/L/K in V4, K432R and S440Q in constant region 4 (C4) and K500R in C5 (Table 2). A minority of viruses isolated at 95 months after SC did not

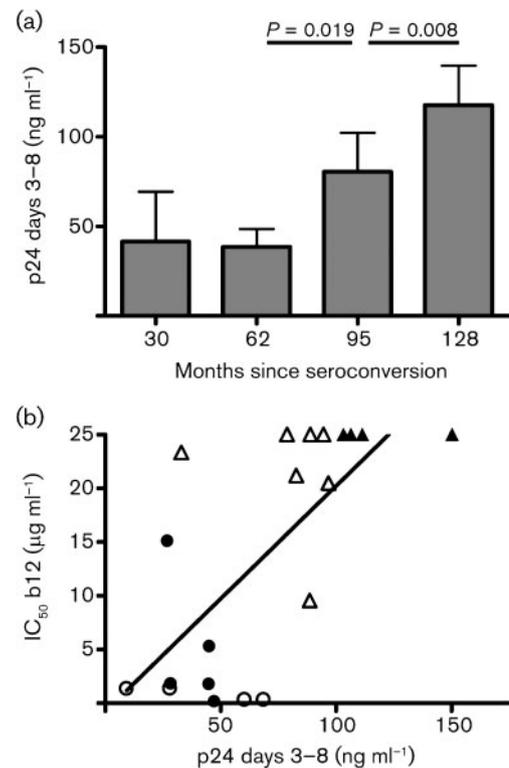


Fig. 3. Relationship between sensitivity to b12 neutralization and replicative capacity of virus variants isolated from patient H5 during the course of infection. (a) Production of p24 between days 3 and 8 after infection of phytohaemagglutinin (PHA)-stimulated PBMCs by chimeric NL4-3 viruses expressing Env of viruses obtained from patient H5 at different time points during infection. Differences in replication kinetics between chimeric viruses containing Env proteins obtained from viruses of different time points were assessed using a *t*-test for independent samples. (b) Correlation between b12 neutralization sensitivity of clonal HIV-1 variants obtained from patient H5 and replication rate for the corresponding chimeric NL4-3/Env viruses from different time points during infection. ○, 30 months; ●, 62 months; △, 95 months; ▲, 128 months. The data were analysed using Spearman's rank test.

contain a substitution at positions 154 (43%), 432 (28%) and/or 500 (14%), but these variants were no longer observed at 128 months after SC. Moreover, sequence variants Q389P and Q389L were only found at 95 months after SC and were completely replaced by variants containing a K at position 389 at the latest time point.

To restrict our study to those amino acid changes that were most likely to have a significant impact on antibody binding or envelope conformation, we decided to focus on the amino acid mutations at positions 154, 178, 389 and 440, and to exclude the minor substitutions K432R and K500R from our subsequent analysis. Interestingly, substitution Q389K was also observed in viruses from the three out of the five other patients in whom b12 neutralization-resistant viruses emerged, but did not seem to be associated with b12 resistance in these viruses. Moreover, late viruses

Table 2. Amino acid substitutions in clonal HIV-1 variants from patient H5 coinciding with increased resistance to b12 neutralization
ND, Not determined.

Months since SC	Virus clone	Amino acid residue at HXB2 position*						IC ₅₀ of b12 (µg ml ⁻¹)
		154	178	389	432	440	500	
30	pLAI	I	K	Q	K	S	K	
	5C6	R	.	0.33
	5D5	R	.	1.35
	5G2	R	.	ND
	6B12	R	.	0.33
	6G1	R	.	1.37
	6G5	K	.	ND
	6H2	R	.	ND
62	7A2	1.80
	7A10	5.28
	7B12	ND
	7D11	ND
	7F2	1.77
	7G2	0.15
	7G5	15.08
	7H6	ND
	8D4	ND
	95	4D3	M	T	P	R	Q	.
4F4		M	T	P	R	Q	R	>25
4F10		.	T	L	.	Q	R	9.58
4F12		M	T	P	.	Q	R	23.35
4G8		.	T	P	R	Q	R	20.49
4G11		M	T	K	R	Q	R	21.19
4H8		.	T	P	R	Q	R	ND
4H10		M	T	P	R	Q	R	>25
128	6C1	M	T	K	R	Q	R	>25
	6C4	M	T	K	R	Q	R	>25
	7A9	M	T	K	R	Q	R	>25
	7E9	M	T	K	R	Q	R	>25
	7G12	M	T	K	R	Q	R	>25

*Compared with the sequence of pLAI, which is given in the top of the table. Amino acid residues identical to the sequence of pLAI at that position are indicated with a dot.

in patient H8 also contained a substitution at position 440 (S440E). However, combinations of the mutations in late viruses from patient H5 were not observed in b12 neutralization-resistant viruses from the other five patients (data not shown).

Mutations at positions 154, 178 and 389 increase b12 neutralization resistance in the background of LAI

The amino acid mutations observed at positions 154, 178, 389 and 440 in virus variants from patient H5 were introduced into the background of LAI to study their effect, alone or in combination, on b12 neutralization sensitivity using a PBMC-based assay. For technical reasons, the amino acid substitution at position 440 was only intro-

duced into LAI as a single mutation and was therefore not analysed in combination with the other substitutions. None of the single mutations at position 154, 178, 389 or 440 resulted in a >2-fold change in resistance to b12 (Fig. 4a). However, viruses containing combinations of the substitutions at positions 154, 178 and/or 389 had a substantially increased resistance to b12 neutralization (>3-fold difference in IC₅₀) compared with wild-type LAI. Resistance to mAb 2G12 was not increased in these variants (data not shown), indicating that the mutations specifically affected the sensitivity to b12 neutralization. The observation that the mutations at positions 154, 178 and 389 in the background of an unrelated virus variant conferred a similar b12-resistant phenotype is supportive for their role in the increased b12 resistance of late virus variants isolated from patient H5.

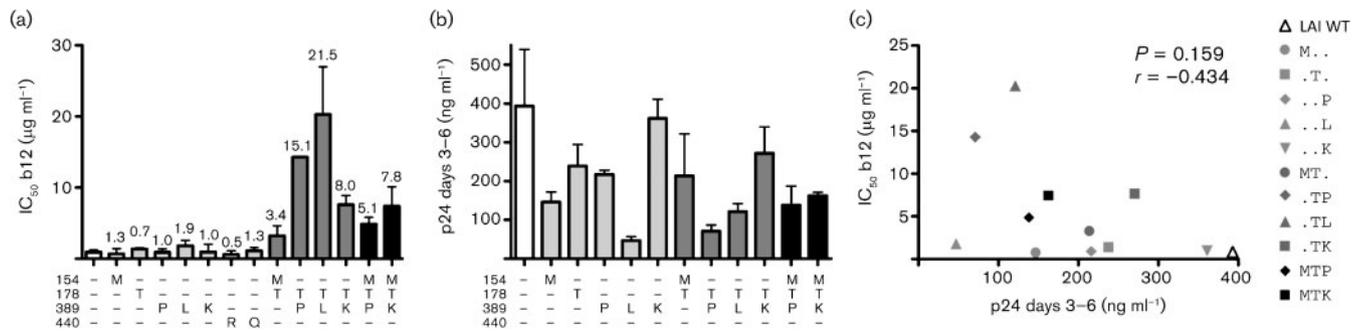


Fig. 4. Effect of mutations in gp120 coinciding with increased b12 neutralization sensitivity and replication rate of virus variants obtained from patient H5, in the background of molecular clone LAI. (a) IC_{50} values for b12 for wild-type LAI (open bar) and single, double and triple LAI mutants (shown as increasingly darker shades of grey) as determined by linear regression. Means \pm SD from two independent experiments are shown. The number presented above each bar indicates the fold change in IC_{50} compared with wild-type LAI. (b) Mean production (\pm SD) of p24 between days 3 and 6 after infection of PHA-stimulated PBMCs by wild-type LAI and mutant LAI variants, as determined by two independent experiments. (c) Correlation between b12 neutralization sensitivity and replication rate for wild-type LAI and LAI mutants. Data were analysed using Spearman's rank test.

Mutations at positions 154, 178 and 389 reduce the replication capacity of LAI

To study whether the mutations at positions 154, 178 and 389, which appeared to play a role in the increased b12 neutralization resistance of viruses from patient H5, may also be involved in the augmented replication kinetics of viruses from H5, we determined the *in vitro* replicative capacity, expressed as the production of p24 between days 3 and 6 after inoculation of PHA-stimulated PBMCs with the various mutant LAI variants. All mutant viruses, with the exception of variant Q389K, showed slower replication kinetics than wild-type LAI (Fig. 4b). Although the replication rate of the viruses did not correlate with their sensitivity to b12 (Fig. 4c), the variants that were most resistant to b12 neutralization (K178T/Q389P and K178T/Q389L) had relatively low levels of replication. Moreover, a trend towards a negative correlation between b12 sensitivity and replicative capacity was observed for the mutant variants with increased resistance to b12 neutralization [i.e. the double and triple mutants ($n=6$); Spearman $r=-0.714$, $P=0.071$]. Thus, the b12-resistant mutations at positions 154, 178 and 389 decreased the viral replicative capacity in the background of LAI, indicating that other as-yet-unidentified compensatory mutations may be involved in the enhanced replication kinetics of late virus variants from patient H5.

b12-resistant mutations in viruses of patient H5 are not selected by cytotoxic T lymphocyte (CTL) pressure

To investigate whether the mutations in the Env protein that were associated with an increased resistance to b12 neutralization could have been selected by CTLs, we predicted epitopes in the gp120 sequence that are restricted by HLA from patient H5. Amino acid residues at positions

178 and 389 were located next to an anchor residue of epitopes predicted to bind to HLA-A*2402. We measured CD8⁺ T-cell gamma interferon (IFN- γ) responses against the peptide epitopes with or without the observed mutations by use of an IFN- γ enzyme-linked immunospot (ELISPOT) assay at different time points during asymptomatic infection, before and after the appearance of virus variants containing the mutation. At none of the time points analysed was a response observed against any of the peptides that were tested (data not shown). Moreover, the predicted score for proteosomal cleavage of the different peptides in the natural processing pathway was low (data not shown), indicating that these peptides are probably not processed and thus not presented by HLA-A*2402. These results suggested that it is highly unlikely that the mutations at positions 178 and 389 in viruses from patient H5 have been selected by CTL pressure.

DISCUSSION

The conserved nature of regions on the viral envelope that are targeted by broadly neutralizing antibodies, such as b12, may be indicative of a limited ability of HIV-1 to escape from these antibodies. However, a significant proportion of primary virus isolates shows resistance to one or more of the currently known broadly neutralizing antibodies (Binley *et al.*, 2004; Quakkelaar *et al.*, 2007b). In this study, we showed that, in seven out of a total of 15 individuals, b12-sensitive viruses that were present early in infection were replaced by virus variants that were resistant to b12 neutralization during the later stages of infection. These observations contrast with previous studies (Keele *et al.*, 2008; Rusert *et al.*, 2005), which may result from the fact that early and late viruses in these studies were not obtained from the same individuals or by our inclusion of viruses from extremely late time points during infection

(112–147 months post-SC), whilst late viruses in the other studies were obtained from chronically infected patients at >24 months after SC.

To understand the emergence of b12-resistant variants during late-stage disease, we investigated potential mechanisms of viral selection. Virus variants resistant to b12 neutralization may be selected as a result of strong humoral immune pressure. However, only four out of seven patients developed cross-reactive neutralizing activity in serum (H3, H6, H8 and H9). Moreover, none of the serum samples from our seven patients showed evidence of CD4-binding site-directed neutralizing activity, indicating that b12-resistant viruses can also be selected in the absence of humoral immune pressure. In addition to a lack of antibody pressure, mutations that were probably involved in the increased resistance to b12 in viruses from patient H5 were not selected as CTL escape variants, as CTL activity against the epitopes in which these mutations were introduced could not be detected. Thus, at least for viruses from patient H5, neither arm of adaptive immunity was involved in the selection of b12-resistant mutations in Env.

In agreement with the absence of immune pressure, the appearance of b12-resistant virus variants relatively late in infection, when host immunity is fading, is also supportive for another selective mechanism. Late-stage CCR5-using virus variants have augmented replication kinetics (Repits *et al.*, 2008), as was also shown here for late-stage virus variants from patient H5. Moreover, they have increased resistance to entry inhibitors (Gray *et al.*, 2005; Koning *et al.*, 2003; Repits *et al.*, 2008) and require lower levels of CD4 expression for cell entry (Gray *et al.*, 2005). In line with these findings, we have shown previously that viruses resistant to b12 neutralization tend to have higher replication kinetics than viruses that are neutralization sensitive to b12 (Quakkelaar *et al.*, 2007a). In four out of seven patients (H3, H4, H5 and H6), the emergence of b12-resistant virus variants coincided with a CD4 count below 200 (data not shown), suggesting that increased b12 resistance may indeed be associated with progressive disease. The adaptation of the virus to the lower availability of CD4⁺ target cells late in infection probably results in changes in the CD4-binding region, which may also affect the binding affinity of b12 to the viral envelope. This hypothesis is supported by the observation that resistance to neutralization by the broadly neutralizing antibodies 2G12, 2F5 and 4E10 was not increased for late virus variants (Bunnik *et al.*, 2009 and data not shown), which indeed suggests that changes in the Env protein resulting in resistance to b12 neutralization specifically involved the CD4-binding site.

In three long-term non-progressors (LTNPs; H7, H8 and H9), CD4 counts were relatively high (>400) at the moment of appearance of b12-resistant virus variants, suggesting that changes in b12 sensitivity in viruses from these patients could not have been driven by reducing numbers of target cells. Interestingly, these individuals were

all heterozygous for the 32 bp deletion in the *ccr5* gene. We have observed recently that viruses in such patients are more resistant to inhibition by β -chemokines, indicating that these viruses have adapted to lower expression levels of CCR5 (D. Edo-Matas, personal communication). Changes in the viral envelope resulting in a more efficient usage of CCR5 will be located primarily in the co-receptor binding site, but might also affect the conformation of the CD4-binding region and thus indirectly the binding and neutralization sensitivity to b12.

As we have not been able to identify a single selection mechanism that may explain the emergence of b12-resistant virus variants in all patients, multiple evolutionary pathways may exist that lead to the same end point. Alternatively, other processes that have not yet been identified may play a role in selection of viruses resistant to b12 neutralization. Moreover, the question remains as to why b12-resistant virus variants do not appear late in infection in all patients. Possibly, the adaptive mechanisms described above do not always result in changes in the viral envelope that also affect the sensitivity of the virus to b12 neutralization.

We detected similar levels of cross-neutralizing activity in serum from some of the progressors (H3 and H6) compared with some of the LTNPs (H8 and H9, respectively), in agreement with recent reports by us (Euler *et al.*, 2010) and others (Piantadosi *et al.*, 2009), which showed that the breadth of the HIV-1-specific humoral immune response is not associated with the clinical course of infection. As the emergence of b12-resistant virus variants was observed in both progressors and LTNPs, it also seems unlikely that the rate of disease progression is influenced by the increased resistance of HIV-1 to b12 neutralization. Differences in disease progression between the patients in this study are therefore more likely to be related to other factors, such as HIV-1 cellular immunity (Kaslow *et al.*, 1996) or the host genetic background (Hogan & Hammer, 2001).

The observation that early virus variants in all seven patients were sensitive to neutralization by b12 suggests that a b12-sensitive phenotype may be favourable for transmission or during the early stages thereafter. As macrophage tropism has been correlated with an increased sensitivity to CD4-binding site-directed agents, including b12 (Duenas-Decamp *et al.*, 2009; Gray *et al.*, 2005; Peters *et al.*, 2008), this may again point to macrophages that are present in the mucosa as one of the first target cells for HIV-1 after transmission (Van 't Wout *et al.*, 1994; Greenhead *et al.*, 2000).

The b12 resistance of late-stage viruses from patient H5 could be mapped to a combination of amino acid residues at positions 154 (in V1), 178 (in V2) and 389 (in V4) in the Env protein. Although these residues are not part of the CD4-binding site, residue 389 is located in the α 4 helix comprising the first section of the V4 loop, which, in the structural model of the unliganded Env, is located in relatively close proximity to the CD4-binding loop (Chen

et al., 2005). Moreover, based on the orientation of the V1V2 stem in crystal structures of Env, it has been suggested that the V2 loop is also close to the Phe⁴³ cavity (Wyatt *et al.*, 1998). Combinations of these substitutions were not observed in b12-resistant viruses from other patients (data not shown), indicating that changes in Env resulting in b12 resistance are virus specific and that different amino acid changes may lead to similar phenotypic alterations. Indeed, other studies have identified various combinations of mutations in regions V2, C3 and/or V4 that conferred resistance to b12 neutralization (Beaumont *et al.*, 2004; Duenas-Decamp *et al.*, 2008; Mo *et al.*, 1997; Poignard *et al.*, 1999). Moreover, whilst individual residues 154M and 389K are observed relatively frequently in subtype B HIV-1 variants in the Los Alamos HIV database (35.1 and 22.1 %, respectively; <http://www.hiv.lanl.gov/content/index>), virus variants in which all three mutations that were present in late viruses from patient H5 (154M + 178T + 389K) have accumulated represent only 1.0 % of all subtype B viruses, indicating that this specific mutational pathway may not be a common way to acquire resistance to b12.

In summary, we have shown that b12-resistant virus variants emerge late in infection in a substantial proportion of HIV-1-infected individuals and that this can occur in the absence of both humoral and cellular immunity. Further research will be needed to reveal common mechanisms by which HIV-1 acquires resistance to broadly neutralizing antibodies such as b12 *in vivo* and to determine which changes in Env account for differences in neutralization sensitivity of the virus.

METHODS

Patient and viruses. The patients in the present study were homosexual male participants of the ACS who were either seropositive at study entry (seroprevalent cases) or who seroconverted during active follow-up. For seroprevalent individuals, an imputed SC date (mean of 18 months before entry into the ACS) was used (van Griensven *et al.*, 1989). All patients were infected with subtype B HIV-1. For better readability, patient identifiers were recoded as H3 (ACH18969), H4 (ACH19768), H5 (ACH19659), H6 (H19298), H7 (H19383), H8 (H19663) and H9 (H19956). Patients H3, H4, H5 and H6 progressed to AIDS within 7–11 years after SC, whilst patients H7, H8 and H9 were LTNP (defined as ≥ 10 years of asymptomatic follow-up with stable CD4⁺ cell counts that were still above 400 cells μl^{-1} in the 9th year of follow-up). Clonal HIV-1 variants were obtained from PBMCs as described previously (Schuitemaker *et al.*, 1992; Van 't Wout *et al.*, 2008). For all clonal HIV-1 variants studied here, CCR5 usage was predicted by the V3 loop sequence (Jensen *et al.*, 2003) and confirmed by the inability of these viruses to replicate in the MT2 cell line. To prevent a change in neutralization sensitivity of the virus variants during *in vitro* culture, the number of PBMC passages of viruses was kept to a minimum (Beaumont *et al.*, 2004).

Cells. Experiments were performed using cryopreserved pooled PBMCs isolated from buffy coats obtained from 10–12 healthy seronegative blood donors by Ficoll-Isopaque density-gradient centrifugation. Cells were thawed and stimulated for 3 days in Iscove's modified Dulbecco's medium (IMDM; Lonza) supplemented

with 10 % fetal bovine serum (FBS; Hyclone), penicillin (100 U ml^{-1} ; Invitrogen), streptomycin (100 $\mu\text{g ml}^{-1}$; Invitrogen), ciproxin (5 $\mu\text{g ml}^{-1}$; Bayer) and PHA (5 $\mu\text{g ml}^{-1}$ Oxoid), at a concentration of 5×10^6 cells ml^{-1} . Subsequently, PBMCs (10^6 cells ml^{-1}) were grown in the absence of PHA, in medium supplemented with recombinant interleukin-2 (20 U ml^{-1} ; Chiron Benelux) and polybrene (5 $\mu\text{g ml}^{-1}$, hexadimethrine bromide; Sigma).

Neutralization assays

(i) PBMC-based assay. Viruses were tested for their neutralization sensitivities against recombinant sCD4 (Progenics), the mAbs b12 (kindly provided by D. Burton, The Scripps Research Institute, CA, USA), 2G12 and 2F5 (Polymun Scientific) and/or patient serum using a PBMC-based assay. From each virus isolate, a final inoculum of 20 TCID₅₀ in a volume of 100 μl was incubated for 1 h at 37 °C with threefold serial dilutions of sCD4 or mAb (range 0.034–25 $\mu\text{g ml}^{-1}$) or serum (range 1:50–1:3200). Subsequently, the mixtures of virus with neutralizing agent were added to 10^5 PHA-stimulated PBMCs in 50 μl medium. Virus production in culture supernatants on day 7 was analysed by an in-house p24 antigen-capture ELISA (Tersmette *et al.*, 1989). The percentage neutralization was calculated by determining the reduction in p24 production in the presence of neutralizing agent compared with the cultures with virus only. Where possible, IC₅₀ values were determined by linear regression.

(ii) U87-based assay. This assay was performed by Monogram Biosciences as part of a larger study (van Gils *et al.*, 2009). The preparation of pseudotyped virus particles has been described in detail elsewhere (Schweighardt *et al.*, 2007). A recombinant virus assay involving a single round of virus infection was used to measure cross-neutralization activity of the sera (Petropoulos *et al.*, 2000; Richman *et al.*, 2003). Diluted pseudoviruses were incubated for 1 h at 37 °C with serial dilutions of the purified serum IgG after which the U87 target cells were added. The ability of patient sera to neutralize virus infection was assessed by measuring luciferase activity 72 h after viral inoculation in comparison with a control infection with a virus pseudotyped with the amphotropic murine leukemia virus Env protein. Neutralization titres were expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50 % (IC₅₀).

Sequence analysis. The *env* genes of clonal HIV-1 variants were amplified from DNA that was isolated from *in vitro*-infected healthy donor PBMCs. The 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 CLUSTAL W in the software package BioEdit (Hall, 1999) and edited manually. The reference sequence of HIV-1 strain HXB2 was included in the alignment to number each aligned residue according to the corresponding position in this reference sequence.

Site-directed mutagenesis and preparation of mutant/chimeric viruses. The *env* fragment from HXB2 nt 5660 (in *vpr*) to 8093 (in *env*) was amplified from pLAI (Peden *et al.*, 1991) by PCR using AccuPrime Taq polymerase (Invitrogen) and cloned into pGEM-T Easy (Promega). Nucleotide substitutions in *env* were introduced using a QuickChange II site-directed mutagenesis kit (Stratagene). The *env* fragments containing single, double or triple nucleotide substitutions were amplified from the pGEM-T Easy vectors by PCR. Full-length LAI mutant viruses were produced by homologous recombination of the *env* PCR products with the original pLAI vector. Briefly, pLAI was restricted with *Sall* (HXB2 nt 5787) and *BsaBI* (HXB2 nt 7560) and subsequently co-transfected 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 ELISA. In a similar fashion, chimeric HIV-1 strain NL4-3/Env viruses were prepared by homologous recombination of *env* PCR products (HXB2 nt 5658–9171) and a pNL4_3 vector (a kind gift from J. Alcamí, Instituto de Salud Carlos III, Spain) restricted with *Xba*I and *Xho*I (at HXB2 nt 6114 and 8898, respectively). The presence of each mutation in LAL, as well as 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 cells) were inoculated with 500 TCID₅₀ of a given HIV-1 variant 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 IMDM supplemented with 10% FBS, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹) and resuspended at a concentration of 10^6 cells ml⁻¹ for culture. Fresh PHA-stimulated PBMCs (10^6 cells) in a volume of 1 ml were added at days 5 and 8. Cultures were maintained for 11 days. Samples (75 µl) for 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 (ml supernatant)⁻¹ by correcting for the differences in volumes of culture supernatant.

IFN-γ ELISPOT assay. IFN-γ-producing, antigen-specific CD8⁺ T cells were measured using an IFN-γ ELISPOT assay with the use of multiscreen, 96-well, membrane-bottomed plates (Millipore) and IFN-γ-specific mAbs (Mabtech). Cryopreserved PBMCs were thawed and suspended in RPMI 1640 containing 10% FBS and incubated at a final concentration of 10^5 cells per well in triplicate. Responses were measured against an excess concentration of 10 µg ml⁻¹ of the following peptides: FYKLDVVPI and mutants FYKLDIVPI and FYTLDIVPI (positions 176–184, mutations underlined), and FYCNSTQLF and mutants FFCNSTLLE, FYCNSTPLF, FYCNSTKLF and FYCNTTKLF (positions 383–391). All peptides were synthesized by the peptide facility at The Netherlands Cancer Institute. PHA stimulation served as a positive control to test the capacity of PBMCs to produce IFN-γ, and medium without peptide or PHA served as a negative control. IFN-γ-producing cells were detected as dark spots and were counted using an A.EL.VIS EliScan (EliAnalyse software, version 4). The number of IFN-γ-producing cells was calculated by subtracting the negative-control value and was reported as the number of spot-forming units per 10^6 PBMCs. Samples with >100 spot-forming units per 10^6 PBMCs, after subtraction of the negative-control values, were considered to be positive.

Statistical analysis. For calculations and statistical analyses, viruses with IC₅₀ values of <0.39 or >25 were assigned a value of 0.20 or 25, respectively. Statistical analyses were performed using the SPSS 16 software package. Differences in sensitivity to b12 neutralization between clonal HIV-1 variants isolated from the earliest two time points and clonal HIV-1 variants isolated from the latest two time points were assessed using a Mann–Whitney *U* test. Differences in replication kinetics between viruses from different time points were evaluated using a *t*-test for independent samples. Correlations between neutralization titres and virus replication capacity were evaluated using Spearman's rank test.

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