Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions
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Emergence of b12 resistant human immunodeficiency virus type 1 variants during natural infection in the absence of humoral or cellular immune pressure
Abstract

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, we studied 15 individuals of whom seven developed b12 resistant viruses late in infection, and 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 was 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, we identified mutations in 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 viral replication kinetics, indicating that selection for enhanced infectivity, possibly driven by the low availability of target cells in later stages of disease, may coincide with increased resistance to CD4 binding site-directed agents, such as b12.

These results show 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.
**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. Nevertheless, neutralizing antibodies (NAbs) directed against such conserved epitopes are considered to be an essential component of a preventive vaccine against HIV-1. Although attempts to induce a potent and broadly neutralizing antibody response have thus far been unsuccessful, a small number of broadly neutralizing monoclonal antibodies have been identified, suggesting that the elicitation of antibodies of similar specificity and breadth by vaccination should be possible.

One of the most promising leads in vaccine development is monoclonal antibody b12, which binds to a conformational epitope on the gp120 subunit that has a distinct overlap with the conserved CD4 binding site. 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. The interaction between b12 and gp120 is centered around the CD4 binding loop spanning residues 364 – 373, but involves many other residues in gp120.

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. 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. These b12 resistant viruses may be selected due to escape from b12 neutralization, as was shown to occur both in vitro and in vivo in the presence of relatively high concentrations of b12 (>10 μg/ml). However, we have recently shown that b12 resistant virus variants emerge during natural infection in the absence of antibody pressure.

In this study, we have identified a total of seven participants of the Amsterdam Cohort Studies, out of a group of 15 individuals who were analyzed, 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 no 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 better understand how b12 resistant viruses may be selected in vivo in the absence of antibody pressure.
Materials and methods

Patient and viruses

The patients in our present study were homosexual male participants of the Amsterdam Cohort Studies on HIV/AIDS (ACS) who were either seropositive at study entry (seroprevalent cases) or who seroconverted during active follow-up. For seroprevalent individuals, an imputed seroconversion (SC) date (on average, 18 months before entry into the ACS) was used. 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, while patients H7, H8, and H9 were long-term non-progressors (LTNP, defined as ≥10 years of asymptomatic follow-up with stable CD4+ cell counts that were still above 400 cells/µl in the 9th year of follow-up). Clonal HIV-1 variants were obtained from PBMC as previously described. For all clonal HIV-1 variants studied here, CCR5 usage was predicted by the V3 loop sequence, 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 peripheral blood mononuclear cell (PBMC) passages of viruses was kept to a minimum.

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 IMDM (Lonza) supplemented with 10% 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 5x10⁵ cells/ml. Subsequently, PBMCs (10⁶/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).

Neutralization assays

(i) PBMC-based assay: Viruses were tested for their neutralization sensitivities against recombinant sCD4 (Progenics, Tarrytown, NY), the monoclonal antibodies b12 (kindly provided by D. Burton), 2G12, and 2F5 (Polymun Scientific, Vienna, Austria), 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 hour at 37 ºC with threefold serial dilutions of sCD4 or monoclonal antibody (range 0.034 - 25 μg/ml), or serum (range 1/50 - 1/3200). Subsequently, the mixtures of virus with neutralizing agent were added to 1x10⁵ PHA-stimulated PBMCs in 50 μl medium. Virus production in culture supernatants on day 7 was analyzed by an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA). The percent neutralization was calculated by determining the reduction in p24
production in the presence of neutralizing agent compared to the cultures with virus only. When possible, 50% inhibitory concentrations (IC₅₀) were determined by linear regression. 

(ii) U87-based assay: This assay was performed by Monogram Biosciences as part of a larger study. The preparation of pseudotyped viral particles is described in detail elsewhere. A recombinant virus assay involving a single round of virus infection was used to measure cross-neutralization activity of the sera. Diluted pseudoviruses were incubated for 1 hour 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 viral infection was assessed by measuring luciferase activity 72 hours after viral inoculation in comparison to a control infection with a virus pseudotyped with the murine leukemia virus envelope (aMLV). Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC₅₀).

Sequence analysis

Envs of clonal HIV-1 variants were amplified from DNA that was isolated from in vitro infected healthy donor PBMC. Env PCR products were subsequently sequenced as described previously. Nucleotide sequences of all virus clones per individual were aligned using ClustalW in the software package of BioEdit, and edited manually. The reference sequence 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 nucleotides (nt) 5660 (in vpr) to 8093 (in env) was amplified from pLAI by PCR using Accuprime Taq polymerase (Invitrogen, Carlsbad, CA) and cloned into pGEM-T-easy (Promega, Madison, WI). Nucleotide substitutions in env were introduced using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). 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. In short, pLAI was restricted with SalI (HXB2 nt 5787) and BsaBI (HXB2 nt 7560) and was subsequently co-transfected with an env PCR product into 293T cells in a 24-wells 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 NL4-3/Env viruses were prepared by homologous recombination of env PCR products (HXB2 nt 5658 to 9171) and a pNL4_3 vector (a kind gift from P. Alcami) restricted with XbaI and XhoI (at HXB2 nt 6114 and 8898, respectively). The presence of each mutation in LAI, as well as the presence of the correct env in NL4-3 was confirmed by sequencing.
Characterization of HIV-1 replication kinetics

2x10^6 PHA-stimulated healthy donor PBMC 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) and streptomycin (100 μg/ml) and resuspended at a concentration of 1x10⁶ cells/ml for culture. Fresh PHA-stimulated PBMC (1x10⁶) in a volume of 1 ml were added at day 5 and day 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 per ml supernatant by correcting for the differences in volumes of culture supernatant.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ-producing antigen-specific CD8⁺ T cells were measured using the IFN-γ ELISPOT assay with the use of multiscreen, 96-well, membrane-bottomed plates (MSIPN4550, Millipore) and IFN-γ-specific monoclonal antibodies (Mabtech). Cryopreserved PBMCs were thawed and suspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and were incubated at a final concentration of 1x10⁵ cells/well in triplicate. Responses were measured against an excess concentration of 10 μg/ml of the following peptides: FYKLDVVPI and mutants FYKLĐI{VPI and FYTLDI{VPI (positions 176-184), and FYCNSTQLF and mutants FFCNSTLLF, 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 10⁶ PBMCs. Samples with >100 spot-forming units/million PBMCs, after subtraction of the negative control values, were considered to have positive results.

Statistical analysis

For calculations and statistical analyses, viruses with IC₅₀ of <0.39 or >25 were assigned a value of 0.20 or 25, respectively. Statistical analyses were performed in 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 titers and viral replication capacity were evaluated using the Spearman’s rank test.
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 3 out of 5 individuals \( ^{20} \). To study whether an increased resistance to b12-mediated neutralization is a relatively common phenomenon for HIV-1 variants in later stages of infection, longitudinally isolated clonal HIV-1 variants from 10 additional participants of the ACS were analyzed for their sensitivity to b12 neutralization using a PBMC-based assay. In seven individuals out of the total of 15 individuals analyzed (47%), virus variants isolated early in infection were sensitive to b12 neutralization, while b12 neutralization resistant viruses emerged later in infection (Figure 9.1, 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), or 4E10, suggesting that this change was specific for b12 (ref. 20, and Supplementary Figure S9.1).

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 potently neutralizing autologous antibodies directed against

Figure 9.1: Sensitivity for neutralization by monoclonal antibody b12 of longitudinally obtained virus variants from seven participants of the ACS

IC\textsubscript{50}s of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC\textsubscript{50}s. Changes in neutralization sensitivity of viruses from the first two time points compared to 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 μg/ml. IC\textsubscript{50}, 50% inhibitory concentration; SC, seroconversion.
the CD4 binding site. Since 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 analyzed the neutralizing activity of sera obtained from these seven patients approximately 4 years after SC against 23 heterologous HIV-1 variants pseudotyped with envelopes from subtypes A, B, C, or D using a U87-based assay. For this tier 2-tier 3 virus panel, consisting of 5 to 7 moderately neutralization sensitive and relatively neutralization resistant variants per subtype, broadly neutralizing serum activity was defined as neutralization of ≥50% of viruses per subtype at serum dilutions higher than 1:100 for at least 3 of the 4 subtypes tested. The neutralizing activity of the sera from our patients against individual viruses from this panel has been reported previously. Overall, sera from 4 out of 7 patients (H3, H6, H8, and H9) exhibited broadly neutralizing activity, while sera from patients H4, H5, and H7 did not (Figure 9.2A). In particular, neutralizing activity in serum from patient H5 was extraordinarily weak, neutralizing none of the heterologous virus variants with an IC₅₀ >100 (Figure 9.2A). We subsequently analyzed the neutralizing activity in serum of our patients against wild-type LAI, which is sensitive to neutralization by b12 and sCD4 (IC₅₀ = 0.95 μg/ml and 1.14 μg/ml, respectively), and against a LAI variant with mutations K178T/Q389P, which shows a >15 fold increase in neutralization resistance to both b12 and CD4 (IC₅₀ = 14.28 μg/ml and >25 μg/ml, respectively), but not to non-CD4 binding site-directed agents (Figure 9.2B). 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 (Figure 9.2B). These results indicate that b12 resistant virus 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 both cross-reactive neutralizing activity, as shown here, as well as autologous neutralizing activity, as shown previously. Moreover, b12 sensitive viruses were rapidly replaced by b12 resistant viruses in this patient, without a period in which sensitive and resistant viruses coexisted 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 infection and of the b12 resistant viruses that had emerged later in infection. To exclude an effect of potential 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 patient H5. For each time point, envelopes from a minimum of 4 and a
Figure 9.2: Heterologous neutralizing serum activity in serum samples 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

(A) Serum neutralization capacity against a panel of 23 heterologous virus variants from different subtypes, expressed as the percentage of virus variants neutralized per subtype. For this panel, broadly neutralizing serum activity is defined as neutralization of at least 50% of the viruses per subtype with IC_{50}s at a reciprocal serum dilution of >100 (as indicated in grey) for at least 3 of the 4 subtypes tested. (B) 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 the fold increase in IC_{50} for LAI TP as compared to LAI WT. Mo, months; SC, seroconversion.

Figure 9.3: Relation 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 day 3 and day 8 after infection of PHA-stimulated PBMC 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 Envs 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. Data were analyzed using the Spearman’s rank test. Mo, months; SC, seroconversion; IC_{50}, 50% inhibitory concentration.
maximum of 7 viruses were analyzed. Chimeric viruses expressing envelopes from variants isolated late in infection (95 and 128 months after SC, respectively) replicated faster as compared to chimeric viruses containing envelopes derived from viruses that were isolated early in infection (30 and 62 months after SC, respectively; Figure 9.3A). Moreover, the higher replication rate was significantly correlated with resistance to b12 neutralization of the original clonal HIV-1 variants isolated from patient H5 (Figure 9.3B; Spearman r = 0.691, P = 0.001).

Table 9.1: Amino acid substitutions in clonal HIV-1 variants from patient H5 coinciding with increased resistance to b12 neutralization

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*As compared to 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.

Aa, amino acid; SC, seroconversion; IC_{50}, 50% inhibitory concentration; n.d., not determined.
Identification of mutations in gp120 of viruses from patient H5 that coincide with increased resistance to b12 neutralization

To identify amino acid (aa) residues in the viral envelope that may play a role in the increased resistance to b12 neutralization and the increased replication kinetics observed for chimeric viruses expressing envelopes from viruses from patient H5, gp120 sequences from variants obtained from the earliest two time points were compared to those from viruses isolated from the last two time points. At six positions in gp120, aa 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 (V) 1, K178T in V2, Q389P/L/K in V4, K432R and S440Q in constant region (C) 4, and K500R in C5 (Table 9.1). A minority of viruses isolated at 95 months after SC did not 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 aa changes that were most likely to have a significant impact on antibody binding or envelope conformation, we decided to focus on the aa 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 3 out of the 5 other patients in whom b12 neutralization resistance viruses emerged, but did not seem to be associated with b12 resistance in these viruses. Moreover, late viruses 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 aa 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 aa substitution at position 440 was only introduced into LAI as a single mutation and was therefore not analyzed in combination with the other substitutions. None of the single mutations at positions 154, 178, 389, or 440 resulted in a >2 fold change in resistance to b12 (Figure 9.4A). 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) as compared to wild-type LAI. Resistance to monoclonal antibody 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...
confer a similar b12 resistant phenotype are supportive for their role in the increased b12 resistance of late virus variants isolated from patient H5.

**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 appear 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 day 3 and day 6 after inoculation of PHA-stimulated PBMC with the various mutant LAI variants. All mutant viruses, with the exception of variant Q389K, showed slower replication kinetics than wild-type LAI (Figure 9.4B). Although the replication rate of the viruses did not correlate with their sensitivity to b12 (Figure 9.4C), 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 resistance mutations at positions 154, 178, and 389 decrease 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.

![Figure 9.4: 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](image)

(A) IC\(_{50}\) for b12 for wild-type LAI (white bar) and single, double, and triple LAI mutants (shown in increasingly darker shades of grey) as determined by linear regression. Average results from two independent experiments are shown. The number presented above each bar indicates the fold change in IC\(_{50}\) as compared to wild-type LAI. (B) Average production of p24 between day 3 and day 6 after infection of PHA-stimulated PBMC 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 analyzed using the Spearman's rank test. Error bars in panels A and B show the standard deviation. IC\(_{50}\), 50% inhibitory concentration.
**b12 resistance mutations in viruses of patient H5 were not selected by CTL pressure**

To investigate whether the mutations in envelope 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 IFN-γ responses against the peptide epitopes with or without the observed mutations by use of the IFN-γ 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 analyzed a response was 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 most likely not processed and thus not presented by HLA-A*2402. These results suggest 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 (BNAbs), 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 BNAbs. In this study, we show that in 7 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 later stages of infection. These observations contrast with previous studies, 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) while 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, serum from none of 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 most likely involved in the increased resistance to b12 in viruses from patient H5 were not selected as CTL escape variants, since 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 resistance 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, as was shown here also for late stage virus variants from patient H5. Moreover, they have an increased resistance to entry inhibitors and require lower levels of CD4 expression for cell entry. In line with these findings, we have previously shown that viruses resistant to b12 neutralization tended to have higher replication kinetics than viruses that were neutralization sensitive to b12. In 4 out of 7 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 most likely results in changes of 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 BNAbs 2G12, 2F5, and 4E10 was not increased for late virus variants (data not shown), which indeed suggests that changes in the envelope resulting in resistance to b12 neutralization specifically involved the CD4 binding site.

In three long-term non-progressors (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 base pair deletion in the ccr5 gene. We have recently observed 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, manuscript in preparation). Changes in the viral envelope resulting in a more efficient usage of CCR5 will primarily be located in the coreceptor 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 endpoint. Alternatively, other processes which have not yet been identified may play a role in selection of viruses resistant to b12 neutralization. Moreover, the question remains why b12 resistant virus variants do not appear late in infection in all patients. Possibly, adaptive mechanisms as 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 to some of the LTNP (H8 and H9, respectively), in agreement with recent reports by us and others which describe 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 viruses variants was observed in both progressors and LTNP, 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 or the host genetic background.

The observation that early virus variants in all seven patients were sensitive to neutralization by b12 suggests that a b12 sensitive phenotype may be favorable 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, this may again point to macrophages that are present in the mucosa as one of the first target cells for HIV-1 after transmission.

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 envelope. Although these residues are not part of the CD4 binding site, residue 389 is located in the α4 helix comprising the first section of the V4 loop, which in the structural model of the unliganded envelop is located in relatively close proximity to the CD4 binding loop. 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. 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 which conferred resistance to b12 neutralization. Moreover, while individual residues 154M and 389K are observed relatively frequent in subtype B HIV-1 variants in the Los Alamos database (35.1% and 22.1%, respectively), 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, which 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 which changes in Env account for differences in neutralization sensitivity of the virus.

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Supplementary Figure S9.1: Sensitivity for neutralization by monoclonal antibodies 2G12, 2F5, and 4E10 of longitudinally obtained virus variants from patients H6, H7, H8, and H9. IC50s of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC50s. Changes in neutralization sensitivity of viruses from the first two time points compared to virus variants from the last two time points were assessed using a Mann-Whitney U test. IC50, 50% inhibitory concentration; SC, seroconversion.