Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions
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Citation for published version (APA):

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Longer V1V2 region with increased number of potential N-linked glycosylation sites in the HIV-1 envelope glycoprotein protects against HIV-specific neutralizing antibodies
**ABSTRACT**

The human immunodeficiency virus type 1 (HIV-1) has the ability to adapt to the host environment by escaping from host immune responses. We previously observed that escape from humoral immunity, both at the individual and at a population level, coincided with longer variable loops and an increased number of potential N-linked glycosylation sites (PNGS) in the viral envelope glycoprotein (Env), and in particular in the variable regions 1 and 2 (V1V2).

Here, we provide several lines of evidence for the role of V1V2 in resistance of HIV-1 to neutralizing antibodies. First, we determined that increasing neutralization resistance of a reference panel of tier-categorized neutralization sensitive and resistant HIV-1 variants coincided with a longer V1V2 loop containing more PNGS. Second, exchange of the different variable regions of Env from a neutralization sensitive HIV-1 variant into a neutralization resistant escape variant from the same individual revealed that the V1V2 loop is a strong determinant for sensitivity to autologous serum neutralization. Third, exchange of the V1V2 loop of neutralization sensitive HIV-1 variants from historical seroconverters with the V1V2 loop of neutralization resistant HIV-1 variants from contemporary seroconverters decreased the neutralization sensitivity to CD4-binding site-directed antibodies.

Overall, we here demonstrate that an increase in length of the V1V2 loop and/or the number of PNGS in that same region of the HIV-1 envelope glycoprotein is directly involved in the protection of HIV-1 against HIV-specific neutralizing antibodies, possibly by shielding underlying epitopes in the envelope glycoprotein from antibody recognition.
**INTRODUCTION**

The HIV-1 envelope glycoprotein (Env) is a major target of the humoral immune response in HIV-1-infected individuals. Antibodies directed against Env can be detected early in infection and are able to neutralize autologous virus variants with increasing titers over time in most patients. HIV-1 Env has developed multiple mechanisms to evade neutralizing antibodies, including the inaccessibility of relevant epitopes due to the trimeric structure of Env, the density of glycosylation and the presence of occluding variable loops on the outer domain of Env. Moreover, some epitopes for neutralizing antibodies only emerge after the conformational changes that occur upon the engagement of Env with the CD4 receptor, when spatial constraints between cell and viral membrane no longer allow binding of the relatively large immunoglobulins to Env.

The HIV-1 Env is synthesized as a gp160 precursor protein, which is subsequently cleaved into two subunits, surface protein gp120 and transmembrane protein gp41. Three subunits of gp120 bind non-covalently to three subunits of gp41 to form a trimeric complex on the surface of the virion. Gp120 is composed of five conserved regions (C1-C5) that are interspersed with five variable regions (V1-V5). The conserved regions form a central core consisting of an inner domain, which interacts with gp41 and is important for trimer formation, and an outer domain, which interacts with the (co)receptors. The variable regions can be highly diverse, both between viruses from different patients and within the viral quasispecies of one patient, and form flexible loop structures on the outer domain of gp120.

Neutralizing antibody pressure results in the rapid selection of escape variants with changes in their variable loops such as large insertions, deletions, and changes in the number of potential N-linked glycosylation sites (PNGS). In particular, length and glycosylation characteristics of the V1V2 loop seem to play a role in resistance against neutralizing antibodies, possibly by shielding underlying regions of Env from antibody recognition and especially in the protection against anti-V3 and anti-CD4-binding site antibodies.

We previously reported on the adaptation of the HIV-1 Env to humoral immunity at a population level, reflected in an increasing resistance of recently transmitted HIV-1 to neutralizing antibodies over a time course of 20 years. The increased neutralization resistance of recently transmitted HIV-1 from contemporary seroconverters, which is most obvious for CD4-binding site-directed antibodies, coincided with changes in the viral envelope, mainly a longer V1 loop with an increased number of PNGS (Euler et al., unpublished data).

In our present study we compared whether these changes in Env are indeed causally related to the differences in neutralization sensitivity of HIV-1 variants. For this reason we first compared molecular characteristics of Envs of reference viruses that are categorized from tier 1 to tier 3 based on their decreasing neutralization sensitivity. We also examined if
changes in the V1V2 loop are directly responsible for increased neutralization resistance by generating chimeric viruses in which Env fragments were exchanged between neutralization sensitive and neutralization resistant HIV-1 variants that were isolated from a single individual early and late in infection, respectively, and between neutralization sensitive and neutralization resistant HIV-1 variants from historical and contemporary seroconverters, respectively. The results from these studies strongly suggest that the V1V2 loop of the envelope glycoprotein is directly involved in the protection of HIV-1 from CD4-binding site-directed neutralizing antibodies, possibly by shielding of the targeted epitopes.

**Material and Methods**

*Viral variants*

The viral variants used to construct the chimeric NL4-3/Env viruses were isolated from HIV-1-infected men who have sex with men of the Amsterdam Cohort Studies (ACS) on HIV and AIDS and were all HIV-1 subtype B. None of the individuals received combination anti-retroviral therapy during the sampling period used for this study. Clonal virus variants were obtained from peripheral blood mononuclear cells (PBMC) as previously described. From patient ACH19642, one virus that was sensitive to autologous neutralization was isolated from PBMC obtained 29 months post-seroconversion (SC) (Genbank GU455427), and one virus that had escaped from autologous neutralization was isolated from PBMC obtained 144 months post-SC (Genbank HQ902005). These virus variants were used to exchange different regions of Env to investigate the effect of these regions on autologous neutralization sensitivity. In addition, 5 recently transmitted viruses from individuals who seroconverted between 1985 and 1989 (historical seroconverters) (Genbank EU43976, EU44098, EU44014, HQ902003 and HQ902004) that were sensitive to neutralization by HIVIg (a pool of purified IgG obtained in 1995 from chronically HIV-1 infected individuals) and 5 recently transmitted viruses from individuals who seroconverted between 2003 and 2006 (contemporary seroconverters) (Genbank HQ901998-HQ902002) that were resistant to neutralization by HIVIg were selected from a previous study to analyze the effect of the V1V2 loop on neutralization sensitivity. To prevent a change in neutralization sensitivity of the virus variants during *in vitro* culture, the number of virus passages in PBMC was kept to a minimum.

The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set out in the declaration of Helsinki and written consent was obtained prior to data collection. The study was approved by the Academic Medical Center institutional medical ethics committee.

*Preparation of chimeric viruses*

Chimeras of gp160 proteins were created using a PCR overlap strategy followed by recombination of the PCR product into a HIV-1 backbone. The variable region inserts
of env and the flanking regions of env were amplified in separate reactions using Expand High Fidelity PCR System (Roche Applied Science). Separate PCR products were then combined by PCR overlap into a chimeric gp160 product that spanned from reference HXB2 nucleotides (nt) 5658 to 9171. All primer combinations are listed in Appendix Table A11.1. Chimeric NL4-3/Env viruses were produced by homologous recombination of the env PCR products with a pNL4-3 vector (a kind gift from J. Alcami). In short, pNL4-3 was restricted with XbaI (HXB2 nt 6114) and XhoI (HXB2 nt 8898) 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 PBMC from healthy seronegative blood donors were added to the culture, and the next day the PBMC 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. The presence of the correct env in NL4-3 was confirmed by sequencing.

PBMC-based antibody neutralization assay
The chimeric NL4-3/Env viruses, in which different regions of Env were exchanged between a virus that was sensitive to autologous neutralization and a virus that had escaped autologous neutralization, were tested for their relative neutralization sensitivity against autologous serum obtained 77 months post-SC and against monoclonal antibody (mAb) 447-52D and TriMab (consisting of mAbs b12, 2G12, and 2F5). The chimeric NL4-3/Env viruses, in which the V1V2 regions were exchanged between virus variants from historical and contemporary seroconverters, were tested for their relative neutralization sensitivities against HIVIg and mAbs VRC01, b12, 447-52D, and 2F5.

PBMC were obtained from buffy-coats from 10 healthy seronegative blood donors and pooled prior to use. Cells were isolated by Ficoll-Isopaque density gradient centrifugation and then stimulated for 3 days in Iscove’s modified Dulbecco medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), ciproxin (5 μg/ml), and phytohemagglutinin (PHA; 5 μg/ml) at a cell concentration of 5x10⁶/ml. After inoculation, the cells (1x10⁶/ml) were grown in the absence of PHA in medium supplemented with recombinant interleukin-2 (20 U/ml; Chiron Benelux, Amsterdam, The Netherlands) and Polybrene (5 μg/ml; hexadimethrine bromide; Sigma, Zwijndrecht, The Netherlands). To prevent possible complement-mediated antibody inhibition of virus infection, complement in human sera and fetal bovine serum was inactivated by a 30 min incubation at 56 °C. From each virus isolate, an inoculum of 20 50% tissue culture infective doses, in a total volume of 50 μl for serum or HIVIg and a total volume of 100 μl for mAbs, was incubated for 1 hour at 37 °C with decreasing concentrations of serum (range, 1:50 – 1:3,200), HIVIg (range, 23 – 1,500 μg/ml), mAb VRC01 (range, 0.078 – 5 μg/ml), TriMab, mAbs b12, 447-52D, or 2F5 (range, 0.03 – 25 μg/ml) in 96-well microtiter plates. Subsequently, 1x10⁵ PHA-stimulated PBMC were added to the mixtures of virus with serum. After 4
hours of incubation with serum or HIVIg, PBMC were washed once in 100 µl phosphate-buffered saline after which fresh medium was added. On day 7, virus production in culture supernatants was analyzed in an in-house p24 antigen capture enzyme-linked immunosorbent assay. Experiments were performed in triplicate. For serum neutralization experiments, background measurements were performed using pooled sera from uninfected individuals. Neutralization sensitivities were expressed as the mAb concentration or reciprocal serum dilution that established 50% inhibition (IC_{50}) of virus infection, as determined by linear regression.

**Sequence analyses**
The gp120 sequences of 116 reference viruses, categorized into tier 1 to tier 3 were obtained from the Los Alamos Database (http://www.hiv.lanl.gov/). Env of clonal HIV-1 variants and chimeric NL4-3 viruses were amplified from DNA that was isolated from in vitro infected healthy donor PBMC. Env PCR products were subsequently sequenced as described previously. The nucleotide sequences of all virus clones were aligned using ClustalW in the software package 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. Potential N-linked glycosylation sites (PNGS) were identified using the N-glycosite tool at the HIV database website (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

**Statistical analyses**
Statistical analyses were performed using the SPSS 16 software package. The differences in amino acid length and number of PNGS between viruses from 3 tier groups was evaluated for statistical significance by a Jonckheere-Terpstra test, while differences between viruses from 2 tier groups were assessed by a Student’s t test. Correlation between neutralization sensitivity and calendar year of isolation of the tier-categorized reference HIV-1 variants was evaluated for statistical significance by Spearman’s correlation. Differences in neutralization sensitivity to HIVIg and mAbs of the V1V2 exchange chimeras as compared to the corresponding NL4-3/Env chimera containing the wild-type Env were evaluated for statistical significance by a Wilcoxon signed rank test.

**Results**
*Env characteristics that coincide with neutralization sensitivity in a reference panel of tier-categorized HIV-1 variants with varying neutralization sensitivity*
We have previously reported that escape from humoral immunity, both at the individual and at a population level, coincides with an increase in envelope glycoprotein length, mainly of the V1V2 loop, and an increase in the number of potential N-linked glycosylation sites (PNGS) within that same region. Here, we first analyzed whether these molecular differences
coincide with HIV-1 neutralization sensitivity in general. To this end, we analyzed the length of Env and number of PNGS in Env in a reference panel of HIV-1 variants that were classified as tier 1 to tier 3 based on their neutralization sensitivity. These reference panels are being used for the assessment of the neutralizing ability of sera and monoclonal antibodies and are supposed to represent genetically and geographically diverse subsets of viruses with neutralization phenotypes that are representative of primary isolates. To better evaluate the neutralizing activity of a serum or monoclonal antibody, a tiered algorithm has been designed in which tier 1 viruses are the most sensitive, and tier 3 viruses are the most resistant to antibody neutralization. A total of 116 reference viruses that were categorized into tier 1 to tier 3 by Seaman et al. and Montefiori et al. (29 tier 1 viruses, 71 tier 2 viruses and 16 tier 3 viruses) were analyzed. We found that the length of Env and the number of PNGS in Env were significantly increased in viruses that were more resistant to antibody neutralization. This increase was most obvious between the tier 2 and tier 3 viruses (Figure 11.1). These differences in length and number of PNGS could be attributed to the variable but not the constant regions of gp120. Of the variable regions, we only observed a significant correlation between neutralization sensitivity and the length and number of PNGS of the Env V1V2 loops (Figure 11.1).

As mentioned above, we have previously reported that HIV-1 variants from contemporary seroconverters were more resistant to neutralization than viruses isolated from individuals infected earlier in the epidemic. To substantiate this finding, we next included the year of isolation of the tier 1-3 categorized reference viruses in our analyses and observed a significant correlation between the level of neutralization resistance and the calendar year of isolation of the tier 1-3 categorized reference viruses (Spearman \( r = 0.369; P = 0.001 \); Figure 11.2). There were 7 viruses with a predicted CXCR4-using phenotype and 2 viruses (HXB2 and BaL) that had a much earlier calendar year of isolation than the other viruses. Exclusion of the CXCR4-using viruses and/or the very early viruses from the analyses had only a minor effect on the correlation between the level of neutralization resistance and the calendar year of isolation (\( r = 0.317; P = 0.007 \), data not shown). Thus, the tier 2 and tier 3 viruses appeared to be isolated at later calendar date in the HIV epidemic than the tier 1 reference HIV-1 variants.

Regions in the envelope glycoprotein that are involved in escape from neutralizing activity in autologous serum

Within a patient, HIV-1 has the ability to adapt to the humoral immune response and to escape from the neutralizing antibodies in serum. Here we examined the effect of changes in different regions of Env on the sensitivity to neutralization by autologous serum through the introduction of gp41 or the variable regions V1, V1V2, V3, or V4 from a neutralization sensitive virus that was isolated early in infection into the envelope background of a virus variant that was isolated late in infection and that had escaped from the neutralizing activity in serum.
Chapter 11

Length

Number of Amino Acids

gp120

p = 0.034

p = 0.021

tier 1 tier 2 tier 3

PNGS

Number of PNGS

gp120

p = 0.012

p = 0.032

tier 1 tier 2 tier 3

variable regions gp120

Number of Amino Acids

p = 0.032

p = 0.025

tier 1 tier 2 tier 3

V1V2

Number of Amino Acids

p = 0.003

p = 0.022

tier 1 tier 2 tier 3

V1

Number of Amino Acids

p = 0.002

p = 0.038

tier 1 tier 2 tier 3

V2

Number of Amino Acids

p = 0.028

tier 1 tier 2 tier 3

V1

Number of PNGS

p = 0.080

tier 1 tier 2 tier 3

V1V2

Number of PNGS

p = 0.007

p = 0.016

tier 1 tier 2 tier 3

V2

Number of PNGS

p = 0.047

tier 1 tier 2 tier 3

V2

Number of PNGS

p = 0.080

tier 1 tier 2 tier 3

V1V2

Number of PNGS

p = 0.007

p = 0.016

tier 1 tier 2 tier 3

V1

Number of PNGS

p = 0.080

tier 1 tier 2 tier 3

V2

Number of PNGS

p = 0.047

tier 1 tier 2 tier 3
Figure 11.1: Envelope glycoprotein characteristics of tier-categorized reference HIV-1 variants
The number of amino acids in the different regions of Env (from top to bottom: gp120, all variable regions, variable regions 1 and 2, variable region 1 and variable region 2) are depicted in the left panels, while the number of potential N-linked glycosylation sites (PNGS) are depicted in the right panels. The tier-categorized reference viruses are grouped according to their neutralization sensitivity, with tier 1 being the most neutralization sensitive and tier 3 the most neutralization resistant. Horizontal bars represent the mean value. The association between number of amino acids or PNGS and neutralization resistance between viruses from 3 tier groups was evaluated for statistical significance by a Jonckheere-Terpstra test and between two tier groups by a Student’s t test.

![Graph showing correlation between neutralization sensitivity and calendar year of isolation of tier-categorized reference HIV-1 variants](image)

Figure 11.2: Correlation between neutralization sensitivity and calendar year of isolation of the tier-categorized reference HIV-1 variants
The tier-categorized reference viruses were ranked by Seaman et al. on the basis of their neutralization sensitivity with the most sensitive ranked 1 and most neutralization resistant ranked 109 as depicted on the y-axis. On the x-axis, the year of isolation of the 109 tier-categorized reference viruses is given. The year of isolation could not be retrieved for 22 tier-categorized reference viruses and these viruses are therefore left out of this analysis. Correlation was evaluated for statistical significance by Spearman’s correlation.

Table 11.1: Neutralizing sensitivity of NL4-3/Env chimeras from a sensitive and resistant virus isolated from one individual

<table>
<thead>
<tr>
<th>Env in chimeric NL4-3/Env virus</th>
<th>IC₅₀ (1/serum dilution)</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autologous serum 77 mo post-SC</td>
<td>HIV-negative serum</td>
</tr>
<tr>
<td>sensitive virus</td>
<td>510</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus with V1 of sensitive virus</td>
<td>837</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus with V1V2 of sensitive virus</td>
<td>680</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus with V3 of sensitive virus</td>
<td>71</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus with V4 of sensitive virus</td>
<td>153</td>
<td>&lt;50</td>
</tr>
<tr>
<td>resistant virus with gp41 of sensitive virus</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

Mo, months; IC₅₀, 50% inhibitory concentration; SC: seroconversion; mAb, monoclonal antibody.
Overall, amino acid sequence variation in Env between these two viruses was 14.5%, with a much higher variation of 76.7%, 44.1% and 38.5% in variable loops V1, V4 and V5, respectively. The V2 loop, V3 loop and gp41 were more conserved with 17.9%, 5.4% and 8.4% difference in amino acid sequence, respectively. The variation in V1 was due to both single amino acid substitutions and an insertion of 7 amino acids in the autologous neutralization resistant virus and these sequence changes also resulted in the introduction of two additional PNGS. Beside these changes, the autologous neutralization resistant virus had six additional PNGS in other regions of Env: one in the V2 loop, two in the C2 region, two in the V4 loop and one in the C4 region. Moreover, the V4 loop in the autologous neutralization resistant virus was 2 amino acids longer than that same loop in the autologous neutralization sensitive virus.

Chimeric NL4-3/Env viruses that harbored the Env of the autologous neutralization resistant virus in which gp41 or one of the variable loops V1, V1V2, V3, or V4 was exchanged with the same region from the autologous neutralization sensitive virus were all replication competent. The reciprocal chimeric NL4-3/Env viruses in which the different regions of Env of the autologous neutralization resistant virus were placed in the background of Env of the autologous neutralization sensitive virus were replication incompetent, despite the correct construction of the chimeric env PCR products as verified by sequencing.

Replacement of the V1 loop or the V1V2 loop of the neutralization escape variant by the shorter, potentially less densely glycosylated loops of the neutralization sensitive virus resulted in a reversion towards a neutralization sensitive phenotype to levels comparable with the original neutralization sensitive virus variant (Table 11.1). Introduction of the V4 loop from the neutralization sensitive virus into the Env background of the neutralization resistant virus resulted in only a small increase in sensitivity to neutralization by autologous

### Table 11.2: Neutralizing sensitivity and env characteristics of viruses from historical (H) and contemporary (P) seroconverters

<table>
<thead>
<tr>
<th>Virus ID</th>
<th>Year of SC</th>
<th>IC\textsubscript{50} (µg/ml)</th>
<th>Length (aa)</th>
<th>PNGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HIVIg VRC01 b12 2F5 447-52D V1 V2 V1V2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19999</td>
<td>1985</td>
<td>831.3 0.121 1.12 &gt;25 &gt;25</td>
<td>23 39 62</td>
<td>4 2 6</td>
</tr>
<tr>
<td>H18766</td>
<td>1988</td>
<td>&gt;1500 0.094 &gt;25 7.29 &gt;25</td>
<td>25 38 63</td>
<td>3 2 5</td>
</tr>
<tr>
<td>H19768</td>
<td>1986</td>
<td>805.4 0.099 1.46 1.51 &gt;25</td>
<td>25 39 64</td>
<td>3 2 5</td>
</tr>
<tr>
<td>H19342</td>
<td>1986</td>
<td>846.3 0.090 1.17 2.17 &gt;25</td>
<td>25 39 64</td>
<td>3 2 5</td>
</tr>
<tr>
<td>H19542</td>
<td>1985</td>
<td>632.9 0.524 1.41 0.69 &gt;25</td>
<td>25 42 67</td>
<td>3 3 6</td>
</tr>
<tr>
<td>P197</td>
<td>2005</td>
<td>1009.0 0.482 &gt;25 0.53 &gt;25</td>
<td>29 39 68</td>
<td>4 2 6</td>
</tr>
<tr>
<td>P180</td>
<td>2004</td>
<td>&gt;1500 0.753 &gt;25 &gt;25 &gt;25</td>
<td>27 41 68</td>
<td>5 2 7</td>
</tr>
<tr>
<td>P004</td>
<td>2003</td>
<td>&gt;1500 0.345 &gt;25 1.76 &gt;25</td>
<td>33 39 72</td>
<td>5 1 6</td>
</tr>
<tr>
<td>P002</td>
<td>2003</td>
<td>1316.3 &gt;5 5.30 0.11 &gt;25</td>
<td>30 46 76</td>
<td>6 5 11</td>
</tr>
<tr>
<td>P127</td>
<td>2005</td>
<td>&gt;1500 &lt;0.078 0.21 4.29 &gt;25</td>
<td>38 68 106</td>
<td>4 5 9</td>
</tr>
</tbody>
</table>

SC, seroconversion; IC\textsubscript{50}, 50% inhibitory concentration; aa, amino acid; PNGS, potential N-linked glycosylation site.
serum, while the exchange of V3 or gp41 had no effect on neutralization sensitivity. A serum pool from HIV-1 negative individuals was used as a negative control for the neutralization assay and did not show any neutralization of the chimeric NL4-3/Env viruses. None of the chimeric NL4-3/Env viruses showed a difference in their sensitivity to TriMab (a 1:1:1 cocktail of mAbs b12, 2G12, and 2F5) as compared to the original autologous neutralization resistant virus. We also tested the neutralization sensitivity of the chimeric NL4-3/Env viruses to mAb 447-52D as an increased sensitivity to this V3-directed antibody may point to unintended conformational changes in the chimeric NL4-3/Env viruses. All original and chimeric NL4-3/Env viruses were resistant to neutralization by mAb 447-52D (Table 11.1), suggesting that exchange of gp41 or variable regions did not change the overall Env conformation of these viruses to expose the V3 loop.

**Effect of V1V2 loops on the neutralization sensitivity of HIV-1 over calendar time**

We previously observed that recently transmitted HIV-1 variants from historical seroconverters were more sensitive to neutralizing antibodies than recently transmitted viruses from contemporary seroconverters and that the viruses from contemporary seroconverters had a longer V1V2 loop and more PNGS in this region than recently transmitted viruses from historical seroconverters 28 (Table 11.2). To determine if these Env characteristics are indeed involved in the neutralization sensitivity of these viruses, we constructed a total of 36 replication competent chimeric NL4-3/Env viruses in which the V1V2 loop was reciprocally exchanged between 5 recently transmitted viruses from historical seroconverters and 5 recently transmitted viruses from contemporary seroconverters. For accurate evaluation of the viral phenotype, all wild type (wt) env genes (n=10) were also cloned into the background of NL4-3. The exchange of V1V2 regions failed for a total of 14 combinations, either due to failure to generate chimeric env PCR products (n=4) or failure to clone the chimeric env PCR products into the NL4-3 background (n=10).

The 36 chimeric NL4-3/Env viruses that were successfully generated, now harboring envelope genes that were chimeric for the V1V2 region, were tested for their sensitivity to neutralization by HIVIg (a pool of purified IgG obtained in 1995 from chronically HIV-1 infected individuals) 42, and mAbs b12, VRC01, 2F5, and 447-52D which was compared to the neutralization sensitivity of the chimeric NL4-3/Env virus with the Env of the original wt virus.

With the exception of chimeric NL4-3/Env-wtH18766, all chimeric NL4-3/Env viruses containing wt Envs of HIV-1 from historical seroconverters were sensitive to neutralization by HIVIg, VRC01, and b12 (Table 11.2). Introduction of a longer V1V2 loop with more PNGS of HIV-1 from contemporary seroconverters into the background of Env of HIV-1 from historical seroconverters increased resistance to HIVIg neutralization to >1,500 µg/ml (highest antibody concentration tested) for 7 out of 18 chimeric NL4-3/Env viruses and raised neutralization resistance to a lesser extend for an additional 6 variants (P = 0.002; Figure 11.3).
For the remaining 5 chimeric NL4-3/Env viruses, neutralization sensitivity to HIVIg did not change as compared to the chimeric NL4-3/Env wt virus. Introduction of the V1V2 region of HIV-1 from contemporary seroconverters into the background of Env of HIV-1 from historical seroconverters resulted in a >2 fold increase in neutralization resistance to mAb VRC01 for 10 out of 18 viruses ($P = 0.001$; Figure 11.3) and to mAb b12 for 11 out of 18 viruses ($P = 0.002$; Figure 11.3). For 6 viruses, an increased neutralization resistance was observed for both VRC01 and b12. In line with our previous observation that the neutralization sensitivity of HIV-1 for 2F5 did not change over the course of the epidemic $^{28}$, sensitivity to neutralization by mAbs 2F5 was similar for viruses with either wt or chimeric $env$. Comparable sensitivities of viruses with wt $env$ and chimeric $env$ to neutralization by mAb 447-52D implicated that the overall confirmation of Env, which may result in enhanced exposure and accessibility of the V3 loop, was intact (Figure 11.3). Replacement of V1V2 in HIV-1 of contemporary seroconverters by the V1V2 loop of HIV-1 from historical seroconverters resulted in a trend towards increased neutralization sensitivity to HIVIg ($P = 0.091$; Figure 11.3), but did not consistently change neutralization phenotypes for mAbs b12, VRC01, and 2F5, as compared to the NL4-3 with the corresponding wt Env from viruses of contemporary seroconverters. We did however observe a significant increase in neutralization sensitivity to mAb 447-52D for these viruses ($P = 0.028$; Figure 11.3), mainly caused by the chimeric NL4-3/Env viruses constructed from wt virus P127.

**DISCUSSION**

HIV-1 has developed multiple mechanisms to evade neutralizing antibodies. The viral envelope glycoprotein can change dramatically during the course of infection due to the positive selection of escape mutations under the immune pressure of neutralizing antibodies. In particular, length and glycosylation characteristics of the V1V2 loop seem to play a role in resistance against neutralizing antibodies, possibly by shielding underlying regions of the envelope glycoprotein from antibody recognition $^{10,15-25}$.

**Figure 11.3: Neutralization sensitivity of the chimeric NL4-3/Env viruses in which the V1V2 loop was exchanged between viruses from historical and contemporary seroconverters**

The chimeric NL4-3/Env viruses consisting of Env of an HIV-1 variant from a historical seroconverter with the V1V2 region of an HIV variant from a contemporary seroconverter are depicted in the left panels and the chimeric NL4-3/Env viruses consisting of Env of an HIV-1 variant from a contemporary seroconverter with the V1V2 region of an HIV variant from a historical seroconverter are depicted in the right panels. IC$_{50}$ values, determined by linear regression, are indicated for HIVIg and mAbs VRC01, b12, 2F5 and 447-52D (from top to bottom), with a gray square representing the original wild type NL4-3/Env virus and each black circle representing a corresponding chimeric NL4-3/Env virus in which the V1V2 loop has been exchanged. Differences in neutralization sensitivity to HIVIg and mAbs of the V1V2 exchange chimeras as compared to the corresponding NL4-3 chimera containing the wild-type Env were evaluated for statistical significance by a Wilcoxon signed rank test. IC$_{50}$ 50% inhibitory concentration.
We recently observed that HIV-1 has become more resistant to neutralizing antibodies at a population level over a time course of 20 years, which also coincided with an increased length of the V1 loop of the HIV-1 envelope glycoprotein and an increased number of PNGS in that same region. These observations implicate a major role of the V1V2 loop of Env in the resistance to antibodies, both at an individual and at a population level. This is further strengthened by our present analysis of envelope gp120 sequences of reference viruses that are categorized from tier 1 to tier 3 based on their susceptibility to neutralization by antibodies and sera. We found that both the length of Env and the number of PNGS in Env were significantly increased in reference viruses that were more resistant to antibody neutralization (tier 3 viruses) as compared to reference viruses that were more sensitive to neutralization (tier 1 and tier 2 viruses). This association was particularly evident for the V1V2 region of Env. Although the association between neutralization sensitivity and both Env length and the number of PNGS in Env was significant for the tier 1 to 3 categorized reference viruses, the variation in envelope characteristics among viruses from each tier was quite large, suggesting that other regions are likely to play a role as well in neutralization sensitivity. In line with our previous observation that HIV-1 is evolving towards a more neutralization resistant phenotype over calendar time, the neutralization resistance of the tier-categorized reference viruses was also correlated with the year of virus isolation, albeit that the correlation was not very strong.

The association between V1V2 loop characteristics and neutralization sensitivity of tier-categorized reference viruses does not provide direct proof for a causal relationship. Results from earlier studies do however implicate a direct role for the V1V2 loop in the resistance to neutralizing antibodies. Replacement of the V1V2 loop of neutralization sensitive T cell line adapted HIV-1 strains or highly neutralization sensitive tier 1 viruses, such as SF162, with the V1V2 loop of a more neutralization resistant virus resulted in a less neutralization sensitive chimeric virus. In our present study, we used primary HIV-1 variants that are representative for the viruses that occur in human infection. However, our results with primary viruses are in full agreement with previous studies, confirming that the V1V2 loop plays an important role in the escape of HIV-1 from neutralizing antibodies over the course of infection. In the patient we studied here longitudinally, the large increase in length of the V1V2 loop and in the number of PNGS in this same region are likely to reflect the large selective pressure on this part of the viral envelope glycoprotein. As we did not map the antibody specificities in the serum of this patient, we cannot conclude whether the selection of these changes is driven by direct escape from V1V2-directed antibodies, or whether the increased size of the V1V2 loops may protect part of the outer domain of gp120 against antibody recognition. In addition, other regions of Env such as V4, which have not been investigated in other studies, may also contribute to the escape of HIV-1 from neutralizing antibodies as we observed here that exchange of the V4 loop also influenced neutralization sensitivity.
Our data illustrate that the longer V1V2 loops with more PNGS on viruses from contemporary seroconverters as compared to those from historical seroconverters are directly related to a decreased sensitivity to neutralization by HIVIg and CD4-binding site-directed antibodies b12 and VRC01. This implicates that longer, more heavily glycosylated V1V2 loops may occlude the CD4-binding site and prevent the binding of CD4-binding site antibodies. Given the relatively large proportion of HIV-1 infected individuals that develop CD4-binding site-directed antibodies 48-50, it seems likely that the adaptation of HIV-1 towards increased neutralization resistance to this antibody specificity is a direct consequence of selective antibody pressure. However, we cannot exclude other mechanisms that may have influenced this process, such as positive selection for HIV-1 variants that have an increased binding affinity to the CD4 receptor. Indeed, changes in the CD4-binding site resulting from competition for binding to the CD4 receptor may at the same time increase sensitivity of HIV-1 to neutralization by CD4-binding site-directed antibodies.

In this scenario, the changes we observed in the V1V2 loops may compensate for the increased exposure of the CD4-binding site to neutralizing antibodies. The observation that neutralization sensitivity to gp41-directed antibodies has not changed over the course of the epidemic 28 indicates that gp41-directed antibody specificities are rare or that they do not provide strong selection pressure on the viral envelope glycosylation. Our data do not discriminate whether a particular glycan or region of the V1V2 loop is involved in the protection against CD4-binding site-directed neutralizing antibodies nor do they exclude the involvement of other regions of the viral envelope.

The Envs of recently transmitted viruses from five historical seroconverters and the Envs of recently transmitted viruses from five contemporary seroconverters showed substantial sequence differences within the V1V2 region but also in other parts of the envelope glycoprotein. Indeed, sequence diversity in the V4 loop of recently transmitted viruses from historical and contemporary seroconverters was observed at 44% of the amino acid positions. Although we did not observe specific variations between recently transmitted viruses from historical and contemporary seroconverters in this region, it cannot be excluded that the mutations in the V4 loop might also have contributed to the increase in neutralization sensitivity. The observation that replacement of the V1V2 loop in Env from HIV-1 of contemporary seroconverters by the V1V2 loop from viruses of historical seroconverters did not result in a significant difference in neutralization sensitivity, might suggests that also other regions or characteristics of Env could play a role in the increase in neutralization resistance.

Despite these unknowns, the results from our study indicate that escape from neutralizing antibodies is mainly mediated by changes in the variable regions of Env and not by mutations in the epitope of the antibodies itself. This is in line with previous observations that escape from neutralizing antibodies and even cross-reactive neutralizing antibodies which are most likely directed against highly conserved regions, does not have a major impact on HIV-1
replication capacity\textsuperscript{23,31,52}. Changing a part of Env, such as the V1V2 loop, might be incompatible with the original conformation of the envelope glycoprotein which may explain why certain chimeric NL4-3/Env viruses lacked replication competence. Conformational changes of Env may influence the neutralization sensitivity of the virus. It has been shown that deletion of the V1V2 loop can result in a more open conformation of the envelope with better exposure of the V3 loop and even relatively small changes in the V1V2 loop may result in a more open envelope conformation, making the virus more susceptible to anti-V3 antibodies\textsuperscript{47,53,54}. Despite the fact that all original viruses had the epitope for V3 antibody 447-52D\textsuperscript{55}, they were all resistant to its neutralizing activity, indicating that the relevant epitope on the V3 loop was not exposed in these viruses. However, some of the chimeric NL4-3/Env viruses were more sensitive to mAb 447-52D suggesting that the envelope conformation of these viruses was more open and no longer occluding the V3 loop. This was most obvious for the chimeric NL4-3/Env variants of the original P127 virus, which were much more sensitive to mAb 447-52D as compared to the original virus. The V1V2 region in virus P127 was at least 30 amino acids longer than the V1V2 loop from the other viruses included in this study, which may explain the effect of V1V2 exchange on envelope conformation.

In conclusion, we here demonstrate that the increased length of the HIV-1 envelope V1V2 loop with an increased number of PNGS is directly responsible for the protection of HIV-1 against CD4-binding site directed neutralizing antibodies, possibly by shielding underlying epitopes in the envelope glycoprotein from antibody recognition. However, our findings do not exclude that changes in other regions of the HIV-1 envelope may cause a neutralization resistant phenotype as well. For vaccine immunogen design, the properties of the V1V2 loop should be taken into account to achieve optimal exposure of certain conserved epitopes. Moreover, as HIV-1 may continue to evolve, it remains to be established whether the epitopes that will be included in a vaccine immunogen will indeed be accessible on HIV-1 variants that will be circulating once a vaccine becomes available.

**Acknowledgements**

This work is financially supported by the Netherlands Organization for Scientific research (NWO), grant number 918.66.628, the European Community’s Six Framework Programme Europrise (FP6/2007-2012) grant number 037611, and the European Community’s Seventh Framework Programme NGIN (FP7/2007-2013) grant number 201433. The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation, the University Medical Center Utrecht, and the Jan van Goyen Clinic are part of the Netherlands HIV Monitoring Foundation and financially supported by the Center for Infectious Disease Control of the Netherlands National Institute for Public Health and the Environment. The funding organizations had no role in study design, data
collection and analysis, decision to publish, or preparation of the manuscript. We thank J. Alcami (Instituto de Salud Carlos III, Spain) for his kind gift of the pNL4-3 vector, D. Burton and A. Hessell (The Scripps Research Institute, USA) for their generous supply of IgG1b12, D. Katinger (Polymun, Austria; as a partner in EUROPRISE) for his supply of mAb 2F5, J. Mascola (Vaccine Research Center, USA) for his supply of VRC01, and S. Zolla-Pazner (New York University Medical Center, USA) for mAb 447-52D. The HIVIg was obtained from North American Biologicals and the National Heart, Lung, and Blood Institute through the US National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The HIV-1 Env TriMab [ARP3240] monoclonal antibodies were donated by D. Katinger and D. Burton, and TriMab was provided by the EU Programme EVA Centre for AIDS Reagents, NIBSC, UK, supported by the EC FP6/7 Europrise Network of Excellence, AVIP and NGIN consortia and the Bill and Melinda Gates sponsored GHRC-CAVD Project.

REFERENCES


### Appendix Table A11.1: Primer combinations for amplification of separate PCR products

<table>
<thead>
<tr>
<th>Region</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>Start to V1</td>
<td>5’-TCCTAGGGATTTGGCTCCATGGGTT-3’</td>
<td>3’-GCAATTTAAGTAACACAGAGTG-5’</td>
</tr>
<tr>
<td>V1 to stop</td>
<td>5’-GGTCCACAGGCCAAAGCCATGG-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
</tr>
<tr>
<td>V2 to stop</td>
<td>5’-TCACTCATCAGGGCAGC-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
</tr>
<tr>
<td>V1V2</td>
<td>5’-GCCGAGGCAAGATCG-3’</td>
<td>3’-GCCGGGGGCCACAATGTTATG-5’</td>
</tr>
<tr>
<td>Start to V3</td>
<td>5’-TCCGTAACAGTTTGCTCCATGGTCTT-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
</tr>
<tr>
<td>V3 to stop</td>
<td>5’-ATGAGACAAGCAATTCATGTAACA-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
</tr>
<tr>
<td>V3</td>
<td>5’-CTGCCCAATTTCTCCGGACATGC-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
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<tr>
<td>Start to V4</td>
<td>5’-TCCAGGATTTGGCTCCATGGTCTT-3’</td>
<td>3’-ATACAGTTGAAATTCCAC-5’</td>
</tr>
<tr>
<td>V4 to stop</td>
<td>5’-CACACTTCTAGCAAGAACATACAC-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
</tr>
<tr>
<td>gp120</td>
<td>5’-TCCAGGATTTGGCTCCATGGTCTT-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
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<tr>
<td>gp41</td>
<td>5’-ACCAAGGGCAAGAAGAG-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGCCTGTTG-5’</td>
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