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van Gils, M.J.

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Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions

Millions of people throughout the world are affected by HIV/AIDS, a devastating disease that is one of the most striking examples of a problem that transcends borders. Global efforts and collaborations in all fields should be made to stop this devastating epidemic; from fundamental research to treatment availability and from education to intervention strategies.

INVITATION

You are cordially invited to attend the public defense of my thesis:

Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions

Friday 1st of April 2011 at 14.00h

At the Agnietenkapel
Oudezijds Voorburgwal 231
in Amsterdam

After the ceremony you are invited to the reception at the Agnietenkapel

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Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions

Marit van Gils
Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college
voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel

op vrijdag 1 april 2011, te 14:00 uur

door

Marit Johanna van Gils

gleboren te Gouda
The research described in this thesis was performed at the Department of Clinical Viro-Immunology, Sanquin Research and Landsteiner Laboratory, and at the Department of Experimental Immunology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

This work was performed as part of the Amsterdam Cohort Studies on HIV-1 infection and AIDS.

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Don't be scared of what you cannot see
Your only fear is possibility
Never wonder what the hell went wrong
Your second chance may never come along

Hold on now your exits here
It's waiting just for you
Don't pause too long
It's fading now
It's ending all too soon you'll see

Pete Murray 'Opportunity'
# Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>General introduction</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>High prevalence of neutralizing activity against multiple unrelated HIV-1 subtype B variants in sera from HIV-1 subtype B infected individuals: evidence for subtype-specific rather than strain-specific neutralizing activity</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>Prevalence of cross-reactive HIV-1-neutralizing activity in HIV-1 infected patients with rapid or slow disease progression</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>Cross-reactive neutralizing humoral immunity does not protect from HIV-1 disease progression</td>
<td>57</td>
</tr>
<tr>
<td>5</td>
<td>Correlations between HIV-1 clades and HIV-1 antibody neutralization sensitivity: significant for vaccine development?</td>
<td>77</td>
</tr>
<tr>
<td>6</td>
<td>Genetic composition of replication competent clonal HIV-1 variants isolated from peripheral blood mononuclear cells (PBMC), HIV-1 proviral DNA from PBMC and HIV-1 RNA in serum in the course of HIV-1 infection</td>
<td>97</td>
</tr>
<tr>
<td>7</td>
<td>Rapid escape from preserved cross-reactive neutralizing humoral immunity without loss of viral fitness in HIV-1 infected progressors and long-term non-progressors</td>
<td>129</td>
</tr>
<tr>
<td>8</td>
<td>Changing sensitivity to broadly neutralizing antibodies b12, 2G12, 2F5, and 4E10 of primary subtype B human immunodeficiency virus type 1 variants in the natural course of infection</td>
<td>151</td>
</tr>
<tr>
<td>9</td>
<td>Emergence of b12 resistant human immunodeficiency virus type 1 variants during natural infection in the absence of humoral or cellular immune pressure</td>
<td>171</td>
</tr>
<tr>
<td>10</td>
<td>Redirected evolution of human immunodeficiency virus type 1 in a patient with cross-reactive neutralizing activity in serum</td>
<td>193</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Chapter 11</td>
<td>Longer V1V2 region with increased number of potential N-linked glycosylation sites in the HIV-1 envelope glycoprotein protects against HIV-specific neutralizing antibodies</td>
<td>205</td>
</tr>
<tr>
<td>Chapter 12</td>
<td>General discussion</td>
<td>227</td>
</tr>
<tr>
<td>Appendix</td>
<td>Summary</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>Samenvatting</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>Dankwoord</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>Curriculum Vitae</td>
<td>261</td>
</tr>
<tr>
<td></td>
<td>Publications</td>
<td>262</td>
</tr>
</tbody>
</table>
General introduction
**General introduction**

**INTRODUCTION TO THE HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1)**

In 1981, a new disease appeared in the human population that was characterized by a deficiency of the immune system. This acquired immune deficiency syndrome (AIDS) was marked by a reduction in CD4+ T-cell numbers and the presentation of unusual infections and cancers. Two years after the recognition of AIDS, the causative agent, an at the time new human retrovirus belonging to the lentivirus family, was identified and named the human immunodeficiency virus type 1 (HIV-1). HIV-1 has been introduced into the human population by cross-species transmissions of the simian immunodeficiency virus (SIV) from non-human primates in West-Central Africa in the beginning of the twentieth century.

Although important progress has been made in the prevention of new HIV-1 infections, and the reduction of the annual number of AIDS related deaths through anti-retroviral therapy, the number of people living with HIV-1 continues to increase and in 2009 approximately 33 million people were infected globally. AIDS-related illnesses remain one of the leading causes of death and are projected to continue as a significant global cause of premature mortality, particularly in developing countries. Despite major advances in the development of antiretroviral treatments and in our understanding of the pathogenesis of HIV-1, the development of a cure, or a vaccine to prevent HIV-1 infection remain enormous scientific challenges.

**HIV-1 INFECTION AND DISEASE COURSE**

HIV-1 spreads through unprotected sexual intercourse, blood-blood contact, or from mother to child during pregnancy, childbirth and breastfeeding. HIV-1 can infect a broad range of immune cells, nevertheless HIV-1 mainly infects CD4+ T-cells, through a multi-step process. In addition to the binding of the CD4 receptor, HIV-1 requires binding to a co-receptor to enter the cell. Chemokine receptors CCR5 and CXCR4 are the most important co-receptors for HIV-1 entry. After entry, HIV-1 integrates into the host-cell DNA, ensuring the replication of HIV-1.

During primary infection high viral load levels can be observed, reaching a peak which is mirrored by a severe loss of CD4+ T-cells from the peripheral blood. Hereafter a decline in viremia can be seen that subsequently settles at a generally lower steady level, the viral setpoint. This decline may be a consequence of an effective immune response and/or due to the limitation of target cells. In the absence of therapy, HIV-1 infected individuals generally develop AIDS within 7-11 years after infection, however the clinical course of HIV-1 infection can be highly variable. Approximately 10-15% of infected individuals are rapid progressors who have a fast CD4+ T-cell decline and who develop AIDS within 3-4 years after infection. Approximately 5-10% of HIV-1 infected individuals are long-term non-progressors (LTNP) who can remain healthy without antiretroviral therapy for more than 10 years. In addition, a small group of individuals known as elite controllers remain to...
have low to undetectable viral loads for at least one year. Both host (for example HLA-B57 and CCR5Δ32) as well as viral factors (for example HIVΔnef) have been associated with slower HIV-1 disease progression.

**HIV-1 Envelope Structure and Function**

Entry of HIV-1 is mediated by the viral envelope glycoprotein (gp) on the surface of the virion. The HIV-1 envelope glycoprotein 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 trimer on the outside 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 between patients as well as within patients, and form flexible loop structures on the outer domain of gp120. When gp120 binds to the CD4 receptor, conformational changes occur in the protein, which reveals the co-receptor binding site that was occluded before CD4 receptor binding. After sequential binding of gp120 to the co-receptor, gp41 mediates membrane fusion and insertion of viral genomic material into the cell.

The chemokine receptors CCR5 and CXCR4 can be used as co-receptor by R5 and X4 HIV-1 strains, respectively. The envelope glycoprotein has developed multiple mechanisms to evade the host humoral immune response, including trimeric exclusion, occluded (co)receptor binding sites, and the shielding of conserved epitopes by the highly variable flexible loops and the presence of many glycans on the outer domain, which reduce the immunogenicity of the envelope glycoprotein.
THE GENETIC DIVERSITY AND EVOLUTION OF HIV-1

One of the characteristics of HIV-1 is its enormous sequence diversity. During infection, each day between $10^8$ and $10^{10}$ viral particles are being produced and eliminated. The error-prone viral reverse-transcriptase enzyme and the lack of proofreading mechanisms during reverse transcription of the viral RNA result in frequent mutations in the viral genome. The large turnover of virus in combination with this high mutation rate results in a mixed population of related but distinct HIV-1 variants, also termed the viral quasispecies. Viral variants within a quasispecies are continuously competing, and the dominant sequence reflects the most fit variant at that time point. After accidental introduction of beneficial mutations in the viral genome or due to changing environmental factors, such as the introduction of antiretroviral agents or the emergence of effective HIV-1 specific immune responses, an initially minor virus population may become dominant, after which a new, so-called population equilibrium is established.

All viral genes are prone to mutation and the proteins they encode are subject to variation. However, large sequence variation is not allowed in each viral genomic region as this may interfere with viral fitness. For example, the gag and pol regions are relatively conserved as viruses with mutations in those regions, which generally come at a fitness cost, are outcompeted by coexisting viruses that lack this mutation. Only when the positive selection pressure on such mutations is higher than the fitness cost associated with it, the mutant virus will be outcompeted by the wild type variants.

The envelope glycoprotein of HIV-1 is highly variable, creating an enormous sequence variation which may be as high as 10% within the viral quasispecies in a single individual. Apparently, the regions in which this huge sequence variation occurs are not critical to the viral replication process.

Despite the high diversity, some viruses are more closely related to each other which has led to a classification of HIV-1 variants into clades, also called subtypes. The main group (M-group) is subdivided into subtypes A to K and different circulating recombinant forms (CRFs), which have different geographic distributions. Subtype B for instance predominates in Europe, the Americas, and Australia, whereas subtype C predominates in Sub-Saharan Africa and the Indian subcontinent. The prevalence of intersubtype recombinant strains is increasing and creates even more HIV-1 genetic diversity. The viral envelope glycoprotein currently already differs by up to 35% between subtypes and up to 20% within subtypes, with the variable regions and also the third constant region (C3) being the most diverse between subtypes.

THE HUMORAL IMMUNE RESPONSE AGAINST HIV-1 IN NATURAL INFECTION

The majority of HIV-1-infected individuals mount an HIV-1-specific neutralizing humoral immune response within weeks to months after primary infection. This response is considered to be strain-specific as neutralizing activity is generally restricted to the
autologous virus variant and mainly directed against the variable regions of the envelope glycoprotein. These antibodies rapidly select for escape variants of HIV-1 that have become resistant to neutralization as a result of amino acid substitutions, insertions and/or deletions in the variable regions, and/or changes in the glycan shield. Escape from neutralizing antibodies may be mediated by mutations in the epitope as a consequence of which the antibody is no longer able to bind, or by changes in other regions of the envelope that prevent access of the antibody to the neutralizing epitope. In response to neutralizing antibody pressure, the envelope glycoprotein can evolve to escape from neutralizing antibodies through variations in the variable loops, including large insertions and 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 the envelope glycoprotein from antibody recognition. Irrespective of the mechanism, such viral escape variants will rapidly be selected by the humoral immune pressure and will replace the neutralization sensitive virus variants (Figure 1.2).

Cross-reactive neutralizing humoral immunity, which can neutralize viruses from different subtypes, may bypass these viral defense mechanisms targeting the more conserved regions on the envelope glycoprotein. However only a few so called broadly neutralizing antibodies, that can neutralize HIV-1 variants from different subtypes, have been isolated from HIV-1 infected individuals. The epitopes of the broadly neutralizing antibodies are conserved domains on the envelope trimer, such as the CD4 binding site, and the membrane proximal external region (MPER) of gp41. These broadly neutralizing antibodies, either alone or in combination, have been shown to give protection from infection after passive transfer in several macaque models. These results together with the high potency of the broadly neutralizing antibodies give hope for a protective vaccine against HIV-1 infection.

Figure 1.2: Escape of HIV-1 from neutralizing antibodies
Neutralizing antibodies are elicited by the viruses present early after infection and rapidly select for antibody escape variants. The emergence of escape variants causes the development of new neutralizing antibodies leading to successive cycles of antibody production and viral escape.
HIV-1 VACCINE DEVELOPMENT

It is generally assumed that an HIV-1 vaccine should elicit both humoral and cellular immune responses. In combination, these responses ideally can protect against acquisition of infection or second best, against disease progression by reducing viral load which will also have an impact on the spread of HIV-1 in the population. Broadly neutralizing antibodies are likely to be a key component of protective vaccine-elicited immunity against HIV-1, however to date, no immunogens have been developed that elicit such broadly neutralizing antibodies.

The design of an immunogen that is capable of eliciting broadly neutralizing antibodies is complicated as the recombinant envelope glycoprotein, even in trimeric form, and vector-expressed HIV-1 envelope glycoproteins do not seem to expose the relevant epitopes. In addition, vaccine-elicited antibodies will have a tough job as HIV-1 seems to be relatively resistant to neutralizing antibodies and is able to rapidly escape from antibody neutralization. Another major obstacle in the development of an effective HIV-1 vaccine is the large sequence diversity, especially of the viral envelope glycoprotein. The nature of neutralizing antibody responses in natural HIV-1 infection may offer new clues for vaccine design. One of the current approaches is the characterization of the epitopes of the very potent broadly neutralizing antibodies that are known to date and to use these epitopes as immunogens to elicit HIV-1 specific neutralizing antibodies with similar potency and breadth.

SCOPE OF THE THESIS

In this thesis, the prevalence, development and characteristics of cross-reactive neutralizing humoral immunity in HIV-1 infected individuals is studied. First, the prevalence of subtype-specific (chapter 2) and cross-reactive neutralizing activity (chapter 3) in serum was studied in 35 participants from the Amsterdam Cohort Studies. Subsequently the impact of cross-reactive neutralizing activity on HIV-1 disease progression was studied in chapter 4. Whether subtype-specific and cross-reactive neutralizing activity are relevant for vaccine development is reviewed in chapter 5.

In chapter 6, the genetic composition of replication competent clonal HIV-1 variants isolated from peripheral blood mononuclear cells (PBMC), HIV-1 proviral DNA from PBMC and HIV-1 RNA in serum is compared at different stages in the course of HIV-1 infection. In chapter 7 the autologous neutralizing antibody response and the escape of HIV-1 from neutralizing antibodies in patients with cross-reactive neutralizing activity is reported.

To further investigate the interaction between HIV-1 and its host we describe the changes in sensitivity to broadly neutralizing monoclonal antibodies b12, 2G12, 2F5 and 4E10 during the course of infection in chapter 8, while chapter 9 focuses in more detail on the changes in sensitivity to b12 neutralization during viral evolution in a patient.
The impact of cross-reactive neutralizing serum activity on viral evolution in a patient is described in chapter 10. Subsequently the adaptation of HIV-1 to humoral immunity, with a focus on the role of the V1V2 loop in the envelope glycoprotein of HIV-1 in the resistance to neutralizing antibodies, is reported in chapter 11.

Finally, in chapter 12 the main results and implications of this thesis are summarized and discussed in the context of current knowledge and HIV-1 vaccine development.

REFERENCES


High prevalence of neutralizing activity against multiple unrelated HIV-1 subtype B variants in sera from HIV-1 subtype B infected individuals: Evidence for subtype-specific rather than strain-specific neutralizing activity
ABSTRACT

It is assumed that an effective HIV-1 vaccine should be capable of eliciting neutralizing antibodies. However, even the best antibodies known to date lack neutralizing ability against a significant proportion of primary HIV-1 variants and despite great efforts, still no immunogen is available that can elicit humoral immunity that can protect against infection or disease progression.

We tested sera from 35 participants from the Amsterdam Cohort Studies on HIV-1 infection, who were all infected with HIV-1 subtype B and therapy naïve at the time of sampling, for neutralizing activity against a panel of 23 tier 2-3 HIV-1 variants, with a minimum of 5 HIV-1 variants per subtype A, B, C and D. Strong cross-clade neutralizing activity was detected in sera from 7 individuals. Strikingly, sera from 22 out of 35 individuals (63%) neutralized 3 or more of the 6 tier 2-3 HIV-1 subtype B viruses in the panel. There was a strong correlation between neutralization titer and breadth in serum. Indeed, the IC$_{50}$ of sera with strong cross-clade neutralizing activity was significantly higher than the IC$_{50}$ of sera with cross-subtype B activity, which in turn had a higher IC$_{50}$ than sera with the lowest neutralization breadth.

These results imply that humoral immunity, at least in HIV-1 subtype B infected individuals, is often subtype-specific rather than strain-specific and that the breadth of neutralization is correlated with the titer of neutralizing activity in serum. Considering the difficulties in designing a vaccine that is capable of eliciting cross-clade neutralizing activity, subtype-specific vaccines may be explored as an interesting alternative.

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**INTRODUCTION**

Neutralizing antibodies (NAb) are believed to be crucial for immunity against viral infections and are therefore considered an essential component of an HIV-1 vaccine elicited immune response. The development of an immunogen that is capable of eliciting NAb is however challenged by the inaccessibility of conserved epitopes and the enormous sequence diversity of the viral envelope which is the main target for NAb. Indeed, the error prone reverse transcriptase, the lack of proofreading, and the extremely rapid viral turnover rate are responsible for huge sequence variation, which can be as high as 10% already within the viral quasispecies in a single individual. This high diversity has led to a classification of HIV-1 variants into distinct clades or subtypes, which are defined as groups of viruses that more closely resemble each other than viruses from other subtypes. The main group (M-group) is subdivided into subtypes A to K and different recombinant forms, which have different geographic distributions; subtype B for instance predominates in Europe, the Americas, and Australia, whereas subtype C predominates in Sub-Saharan Africa. The viral envelope currently differs by up to 35% between subtypes and up to 20% within subtypes. The enormity of this challenge can be put into perspective by comparison with the influenza vaccine, where a diversity of less then 2% in amino acid changes can already cause failure in the cross-reactivity of the polyclonal response elicited by the vaccine. It may therefore be put into question whether a single vaccine capable of eliciting neutralizing antibodies against all HIV-1 variants is feasible.

In addition to the high sequence diversity, the humoral immune response is thwarted by the inaccessibility of the relevant (conserved) epitopes. The inaccessibility of relevant epitopes on the HIV-1 envelope is due to a high level of glycosylation, occlusion within the oligomeric structure of the viral envelope, and the fact that their formation occurs only after engagement of the viral envelope with CD4, when spatial constraints do not allow binding of the relatively large immunoglobulins. Despite the viral mechanisms for evading humoral immunity, HIV-1 does elicit neutralizing antibodies in the natural course of infection. These however are considered to be mainly strain-specific, so only capable of neutralizing autologous virus variants and their epitopes are therefore considered irrelevant for vaccine design.

Broadly neutralizing antibodies (BrNAb) may bypass the viral defense mechanisms as they have the ability to neutralize HIV-1 variants from different subtypes. Four well known BrNAbs, b12, 2G12, 2F5 and 4E10, have been isolated from HIV-1 infected individuals. One of the current vaccine strategies is to design an immunogen that mimics the epitopes of these broadly neutralizing antibodies. However, an effective vaccine would require additional epitope specificities, as a significant proportion (~15%) of primary subtype A, B, C, D, and CRF01-AE is resistant to neutralization by all 4 BrNAb mentioned above. The high sequence diversity between HIV-1 variants may underlie the incomplete coverage by BrNAb. In that light, vaccine elicited subtype specific neutralizing antibodies may be the
best alternative to BrNAb. However, the existence of HIV-1 neutralization serotypes has been questioned. Here, we studied the breadth of serum neutralizing activity in 35 HIV-1 subtype B infected individuals. We found that sera from 7 individuals had highly cross-clade neutralizing activity, and that the majority of sera neutralized multiple unrelated subtype B HIV-1 variants, providing evidence for a HIV-1 subtype B neutralization serotype.

**Materials and Methods**

*Patients*

The study group consisted of long-term non-progressors (LTNP; defined as HIV-1 infected individuals who have ≥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) and progressors (HIV-1 infected individuals who progressed to AIDS within 7 years after (imputed) seroconversion) who were all participating in the Amsterdam Cohort Studies on HIV and AIDS in homosexual men. All individuals were infected with HIV-1 subtype B, and were either seropositive at entry in the cohort studies (seroprevalent cases with an imputed SC date on average 18 months before entry in the cohort) or seroconverted during active follow-up in the cohort studies. None of the participants received combination anti-retroviral therapy during the sampling period; samples were obtained on average at 28 months (range 24-33 months). 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 from each participant. The study was approved by the Academic Medical Center institutional medical ethics committee.

*Viruses*

Sera from all 35 patients were tested for neutralizing activity in a pseudovirus assay developed by Monogram Biosciences. The tier 2-3 virus panel that we used for determining cross-neutralizing activity in serum consisted of HIV-1 pseudoviruses from subtypes A (n=5), B (n=6), C (n=7), and D (n=5) and included recently transmitted isolates, and moderately neutralization sensitive and resistant primary HIV-1 variants, based on previously determined neutralization sensitivities to subtype B sera and MAbs b12, 2G12 and 4E10. In addition, 5 subtype B HIV-1 reference strains were included (1196, Bal, JR-CSF, NL4-3 and SF162). Pseudotyped viral particles were produced by cotransfecting HEK293 cells with an expression vector carrying the patient-derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucPCND-ΔU3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudovirus stocks were then diluted to titers that, as measured by relative light units, fell within a range known to yield reproducible IC₅₀/s.
Neutralization assay
A recombinant virus assay involving a single round of virus infection was used to measure neutralization. Diluted pseudoviruses were incubated for 1 hour at 37 °C with serial dilutions of serum 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). Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control.

Statistical analyses
Statistical analyses were performed using the SPSS 16 software package. Neutralization titers, expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC50), and the number of viruses that were neutralized were not normally distributed. Therefore the non-parametric Kruskal-Wallis test and Mann-Whitney U test were used to compare the neutralization titers between sera that had strong cross-clade neutralizing activity, only cross-subtype B specific neutralizing activity, or no cross-reactive neutralizing activity at all. For the calculation of IC50 values for viruses that were not inhibited by the 1:40 serum dilution we assumed that 50% inhibition would have occurred at a 1:20 serum dilution. A result was considered significant when the P value was <0.05.

Results
Prevalence of strong cross-clade HIV-1 specific neutralizing activity in patient sera
We studied sera from 35 participants from the ACS for the breadth and titer of HIV-1 specific neutralizing activity. Serum samples were obtained between 24 and 33 months after the estimated day of seroconversion and all participants were therapy naïve at this point. HIV-1 specific neutralizing antibody activity was measured in a cell-based infectivity assay using recombinant viruses that carried a luciferase reporter gene and that were pseudotyped with envelope proteins from tier 2-3 HIV-1 subtype A, B, C, and D. For comparison, 5 HIV-1 subtype B reference strains were additionally tested. To monitor neutralizing activity not mediated by antibodies directed against HIV-1 Env-specific antibodies, each plasma sample was also tested against a recombinant virus stock that was pseudotyped with amphotropic murine leukemia virus envelope proteins (gp70SU and p15TM). Typically, neutralization titers, expressed as the reciprocal dilution of plasma that established 50% inhibition (IC50) of virus infection, were <40 for amphotropic murine leukemia virus (aMLV) controls. No differences in neutralizing activity were observed between sera from LTNP and progressors (cross-clade neutralizing activity in 3/20 LTNP and 4/15 progressors) (van Gils et al. AIDS in press). In all sera, neutralizing activity against the reference strains was observed (Figure 2.1).
Neutralizing activity in sera from participants of the ACS was measured against 23 pseudoviruses, HIV-1 subtypes A, B, C, D, and O. The neutralizing activity was categorized as follows:

- **Strong cross-reactive neutralizing activity** (≥50% of viruses per subtype with IC50 ≥ 100 for ≥3 subtypes) - n = 7
- **Cross-reactive neutralizing activity against multiple subtype B variants but minimal neutralizing activity against other subtypes** (≥50% of subtype B viruses neutralized) - n = 15
- **Absent cross-reactive neutralizing activity** - n = 13

Neutralization titers were calculated as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC50). Neutralization titers that were 3 times greater than the negative control (aMLV) were indicated in gray. IC50 < 40 were indicated with a stripe. LTNP patient ID numbers were indicated in gray. n.d. indicates not done.

![Table of neutralization titers](image-url)
Strong HIV-specific cross-clade neutralizing activity, defined as an IC$_{50}$ ≥ 100 for at least 50% of the tier 2-3 viruses from at least 3 different subtypes (so excluding the reference strains), was observed in sera from 7 of 35 individuals (20%) (Figure 2.1). Interestingly, sera from three of these individuals neutralized >80% of all the tier 2-3 viruses in the panel with an IC$_{50}$ ≥ 100 (Figure 2.1; patients 19298, 19642 and 19708).

Prevalence of sera with cross-reactive neutralizing activity against multiple HIV-1 subtype B variants but less to viruses from other subtypes

The sera from 7 individuals with strong cross-clade neutralizing activity also neutralized 5 to 6 out of the 6 tier 2-3 subtype B HIV-1 variants in the panel. Sera from the other 28 of 35 HIV-1 subtype B infected individuals studied here lacked strong cross-clade neutralizing activity against HIV-1 variants from multiple subtypes, according to the definition described above. Interestingly, while sequence diversity between the envelope genes of the tier 2-3 HIV-1 subtype B variants in the panel, so again excluding the reference strains, varied by up to 12%, and while phylogenetic analysis did not reveal clustering of the viruses from this panel with autologous viruses of the different patients studied here (data not shown), sera from 26 of these 28 patients (93%) who lacked strong cross-clade neutralizing activity, showed neutralizing activity against at least 1 of the 6 unrelated tier 2-3 HIV-1 subtype B variants in the panel (Figure 2.1). Strikingly, sera from 15 of these 28 patients (54%) neutralized even 3 or more of the 6 unrelated tier 2-3 HIV-1 subtype B variants in the panel (Figure 2.1). Interestingly, 4 of these patients (19250, 19559, 19663, 19768) and also 2 patients with strong cross-clade neutralizing activity (18969, 19829) showed the same breadth of neutralization against subtype B and subtype C viruses with even higher neutralizing titers against the subtype C variants than against the subtype B variants.

The breadth of neutralizing activity against viruses from the other 3 subtypes was significantly lower, in agreement with the fact that these sera did not have strong cross-clade neutralizing activity. These data show that apart from the 7 sera with strong cross-clade neutralizing activity, the majority of sera had neutralizing activity against multiple and diverse subtype B HIV-1 variants. Indeed, of the total of 35 individuals, 22 individuals (63%) had neutralizing activity against at least 3 of the tier 2-3 subtype B viruses in the panel.

Correlation between titer and breadth of HIV-1 specific neutralizing activity in serum

Characteristics of heterologous HIV-1 specific neutralizing serum reactivity are not known in great detail. Here, we observed a strong correlation between the titer of neutralizing activity and the number of different viruses that were neutralized by a serum (Figure 2.2). Indeed, for neutralization of each individual virus in the panel of tier 2-3 HIV-1 subtype B viruses, the mean IC$_{50}$ values were significantly higher for sera that had strong cross-reactive neutralizing activity against viruses from different subtypes (Figure 2.2A, white bars) as compared to sera with cross-reactive neutralizing activity against multiple subtype B variants.
but not against viruses from other subtypes (Figure 2.2A, grey bars). Additionally, sera from the latter group had in turn a significantly higher mean neutralizing titer against 4 of the 6 tier 2-3 subtype B HIV-1 variants in the panel (92BR020, APV-16, APV-20, and MB_pB1) as compared to the mean neutralizing titers in the 13 patient sera that neutralized ≤ 2 of the HIV-1 subtype B viruses in the panel (Figure 2.2A, dashed bars).

The mean neutralizing titers in the patient sera studied here were higher for some of the reference viruses that were used in this study (1196, Bal, JR-CSF, NL4-3 and SF162; Figure 2.2B), in agreement with the generally higher neutralization sensitivity of these viruses. Interestingly, also for these reference strains, we observed the same pattern between neutralization breadth and titer. Indeed, the mean neutralizing titer of the 7 sera with strong cross-clade neutralizing activity (Figure 2.2B, white bars) was significantly higher for each individual reference virus than the mean neutralizing titer in the 15 sera with subtype B specific cross-reactive neutralizing activity (Figure 2.2B, grey bars) while the mean neutralizing titers in these sera were again higher than the mean neutralizing titer in the 13

Figure 2.2: Correlation between titer and breadth of HIV-1 specific neutralizing humoral immunity in sera of HIV-1 infected individuals

Mean neutralizing titer of sera in defined groups, according to their ability to neutralize the tier 2-3 viruses from the panel, against 6 unrelated tier 2-3 subtype B HIV-1 variants (A) and 5 subtype B reference strains (B). The patient sera were grouped based on neutralizing activity against the tier 2-3 viruses, excluding the neutralizing activity against the reference strains: strong cross-clade neutralizing activity, Figure 2.1 (≥50% of viruses per subtype with IC_{50} ≥ 100 for ≥3 subtypes, n=7), cross-reactive neutralizing activity against multiple subtype B variants but minimal neutralizing activity against other subtypes (≥50% of subtype B viruses neutralized, n=15), or absent cross-reactive neutralizing activity (n=13). Serum neutralizing titers required for 50% inhibition of the tier 2-3 HIV-1 subtype B virus variants in the panel were calculated. White bars: sera with strong cross-clade neutralizing activity (n=7); grey bars: sera with cross-reactive neutralizing activity against multiple subtype B variants but minimal reactivity against viruses from other subtypes (n=15); dashed bars: sera that lack cross-reactive neutralizing activity (n=13). Neutralizing titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50%. Significant difference between the three groups were indicated; * P < 0.05, ** P < 0.01, *** P < 0.001 (Mann-Whitney U test).
sera that lacked cross-reactivity (Figure 2.2B, dashed bars). For JRCSF, a tier 2 reference strain with a known neutralization resistant phenotype, the mean neutralizing titer in the 15 sera with cross-subtype B activity was similar to the mean titer in the 13 sera that lacked cross-reactive neutralizing activity (Figure 2.2B).

The neutralizing titers against viruses of subtype A, C and D also showed a correlation with the neutralization breadth against these viruses, albeit that the differences in titers between groups of sera with strong cross-clade neutralizing activity, cross-subtype B neutralizing activity or almost absent neutralizing activity were less strong (data not shown).

**Discussion**

All vaccines that provide protection against viral infections elicit at least a potent humoral immune response. In line, HIV-1 vaccine research is aiming for an immunogen in which epitopes for broadly neutralizing antibodies are present. This is a challenging task as the HIV-1 envelope has evolved towards a structure in which the relevant epitopes are absent in the native protein, occluded in the oligomeric structure, and/or covered by N-linked glycosylation sites. In addition, the HIV-1 envelope gene is highly variable. This variation, which can be up to 35% between different subtypes, makes it unlikely that a single vaccine will be capable of eliciting a humoral immune response that would cover protection against all possible variants. Indeed, even the best broadly neutralizing antibodies known to date do not neutralize all of the circulating HIV-1 variants. Most HIV-1 infected individuals mount an HIV-1 specific humoral immune response but these antibodies are considered strain-specific as neutralizing activity is assumed to be limited to the autologous virus strain. Indeed, the majority of HIV-1 infected individuals do not develop cross-clade neutralizing activity that is capable of neutralizing HIV-1 variants from different subtypes. However, cross-reactive neutralization of different HIV-1 variants of the same subtype has received only little attention.

The findings of our present study suggest that subtype-specific differences in HIV-1 neutralization may exist, similar to what is known for influenza virus. Overall, we observed that sera from HIV-1 subtype B infected individuals had stronger neutralizing activity against multiple unrelated HIV-1 subtype B variants with substantial sequence diversity in their envelopes, than against HIV-1 variants from subtype A, C and D. However, sera from 4 patients with neutralizing activity against multiple subtype B variants and from 2 patients with strong cross-clade neutralizing activity, had higher neutralizing titers against the subtype C variants in our panel than against the variants from the other subtypes, including subtype B. This may suggest that at least some of the epitopes on the envelope of subtype B variants that elicited cross-clade neutralizing activity may be even better exposed on subtype C variants.

Obviously, it remains to be established whether this observation holds also true for sera from individuals infected with other HIV-1 subtypes. Other studies have not provided...
evidence for HIV-1 subtype-specific differences in HIV-1 neutralizing activity in serum. However, these studies were performed with only a limited number of HIV-1 variants and sometimes with a pool of patient sera in which different neutralizing epitope specificities may have been mixed. Moreover, these studies strongly focused on broadly neutralizing antibodies that by definition neutralize HIV-1 variants from different subtypes. Although not specifically emphasized by the authors, some previous reports do include data that show that neutralizing activity in patient sera was stronger against viruses that were from the same subtype as the autologous virus.

The exact nature of the epitopes at which cross-clade neutralizing activity and subtype-specific cross-reactive neutralizing activity is directed remains to be established. It was recently reported that cross-clade neutralizing activity is not only directed against the conserved regions of the envelope, such as the CD4 binding site or the V3 loop. It is likely that epitopes that are less well conserved between subtypes but conserved within a subtype are capable of eliciting subtype-specific cross-reactive neutralizing activity. Alternatively, the neutralizing activity is mediated by antibodies directed against the V3 loop, similar to the HIV-1 subtype B specific neutralizing activity of the well characterized monoclonal antibody 446-52D. This NAb recognizes a GpXR motive which is very well conserved in the V3 loop of subtype B HIV-1 variants.

The observation that subtype-specific neutralizing activity in serum may exist can provide a new lead in HIV-1 vaccine development. Indeed, the high sequence diversity between HIV-1 variants of different subtypes may stand in the way of the development of a single vaccine capable of eliciting neutralizing humoral immunity against all circulating HIV-1 variants. Obviously, this approach may be considered once a successful protein vaccine has been developed, which is a major challenge by itself.

Interestingly, we observed relatively strong cross-reactive neutralizing activity against multiple subtype B variants in sera from 63% of subtype B-infected individuals studied here, suggesting that the epitopes that have elicited these humoral responses are present and accessible on natural HIV-1 variants. Although HIV-1 may rapidly escape from this antibody pressure, escape may be prevented if a vaccine elicits sterilizing immunity which is capable of completely preventing viral replication.

We have also observed that the ability of serum to neutralize different viruses is directly related to the neutralization titer in serum (modeled in Figure 2.3). Although this finding does not exclude that highly potent antibody specificities may exist at an average concentration in serum, as was recently reported for 2 novel cross-clade neutralizing antibodies PG9 and PG16, it may imply that sera with highly cross-clade neutralizing ability in general harbor multiple epitope specificities or that a high quantity of a single antibody specificity is more potent, even against unrelated HIV-1 variants. This observation indicates that in general, optimal boosting during vaccination, to increase the antibody titer elicited by a future vaccine may also significantly increase the breadth of the neutralizing activity.
In conclusion we have found evidence for subtype-specific neutralizing activity and a positive correlation between the titer and breadth of neutralizing activity in patient sera. The design of improved adjuvants that can optimize humoral immune responses, in combination with potentially subtype-specific epitopes, may thus provide new leads on the way to a potent HIV-1 vaccine. Developing and administering multiple HIV vaccines is far less ideal than having a single vaccine that would cover all circulating HIV variants. However, design and delivery of a single vaccine that is capable of eliciting potent and cross-clade neutralizing immunity against HIV-1 have not yet been successful. Although we realize that probably any vaccine approach will have to deal with the complexity of the HIV-1 envelope molecule and the difficulty to mimic it as an immunogen, based on our data we suggest that the approach of subtype-specific vaccines may be worthwhile to consider in current strategies.

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REFERENCES


HIV-1 subtype B neutralizing humoral immunity
Prevalence of cross-reactive HIV-1-neutralizing activity in HIV-1 infected patients with rapid or slow disease progression
ABSTRACT

The native envelope gp160 trimer of HIV-1 is thought to shield vulnerable epitopes that could otherwise elicit effectively neutralizing antibodies. However, little is known about the prevalence of naturally occurring broadly neutralizing activity in serum of HIV-1 infected individuals.

Here we studied 35 participants of the Amsterdam Cohort Studies on HIV-1 infection (20 long-term non-progressors (LTNP) and 15 progressors) for the presence of cross-reactive neutralizing activity in their sera at 2 and 4 years after seroconversion (SC). Neutralizing activity was tested in a pseudovirus assay, against a panel of HIV-1 envelope variants from subtype A, B, C, and D.

Already at year 2 post-SC, 7 out of 35 individuals (20%) had cross-reactive neutralizing activity, which increased to 11 individuals (31%) at 4 years post-SC. There was no difference in the prevalence of cross-reactive neutralizing serum activity between LTNP and progressors. Interestingly, high plasma viral RNA load and low CD4+ T cell count at set-point were associated with early development of cross-reactive neutralizing activity. Neutralization titers in serum increased during the course of infection for 91% of individuals studied here, albeit less rapidly for those who did not develop cross-reactive neutralizing activity.

Overall, we here demonstrate a relatively high prevalence of cross-reactive neutralizing serum activity in HIV-1 infected individuals, which increased with duration of infection. These data may imply that immunogenicity of the native envelope spike of HIV-1 for eliciting cross reactive humoral immune responses may be better than previously anticipated.
INTRODUCTION

The ability to elicit potent and cross-reactive neutralizing human immunodeficiency virus type 1 (HIV-1) specific humoral immunity is one of the major goals in HIV-1 vaccine development. One of the current approaches is the characterization of the epitopes of the best broadly neutralizing antibodies (BrNAb) that are known to date and to use these epitopes as immunogens to elicit HIV-1 specific neutralizing antibodies with similar potency and breadth. However, antibodies elicited by currently available HIV-1 envelope based immunogens do not display potent and cross-reactive neutralizing activity. Interestingly, the BrNAb b12, 2G12, 2F5, and 4E10, have all been isolated from HIV-1 infected individuals suggesting that the native envelopes of the HIV-1 variants in these individuals were capable of eliciting these antibody responses.

Despite intense research efforts, the number of isolated BrNAb has remained low, which has led to the assumption that BrNAb are rare in natural HIV-1 infection. However, several recent studies have shown cross-reactive neutralizing HIV-1 specific neutralizing activity in sera from various HIV-1 infected patients. In three studies the specificity of the neutralizing activity was identified, but it is still unclear if the breadth of the neutralizing activity in serum is determined by a single high affinity antibody directed against a highly conserved epitope in the envelope protein, or if it is the combined effect of multiple co-existing neutralizing antibodies directed at multiple distinct regions of the envelope. In line with both possibilities is the observation that cross-reactive HIV-1 specific neutralizing activity in serum develops over time. Indeed, early in infection, neutralizing activity in serum is directed against autologous HIV-1 variants and rarely directed against heterologous isolates, while plasma's collected during the chronic phase of infection display various degrees of cross neutralizing activities although a more exact prevalence of cross-reactive neutralizing activity in sera from HIV-1 infected individuals remains to be established.

To support HIV-1 vaccine development, more insight is needed into factors that are associated with the ability of the host to elicit a cross-reactive neutralizing humoral immune response, and how such a neutralizing serum response evolves over time. Here we studied the potency and breadth of HIV-1 specific neutralizing humoral immunity in serum samples that were obtained at 2 and 4 years after seroconversion from 35 participants of the Amsterdam Cohort Studies. The prevalence of cross-reactive neutralizing activity in serum in our study group was 31%. We observed a strong correlation between duration of infection and breadth of the neutralizing HIV-1 specific humoral immune response, and a high plasma viral RNA load set-point and low CD4+ T cell count set-point were both associated with the early development of cross-reactive neutralizing activity. However, the prevalence of cross-reactive neutralizing activity in serum was similar for LTNP and progressors, excluding a correlation between potent humoral immunity and the clinical course of infection.
**Materials and Methods**

**Patients**

The study group consisted of long-term non-progressors (LTNP; defined as HIV-1 infected individuals who have ≥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) and progressors (HIV-1 infected individuals who progressed to AIDS within 7 years after (imputed) seroconversion (SC)) who were all participating in the Amsterdam Cohort Studies (ACS) on HIV and AIDS in homosexual men. All individuals were infected with HIV-1 subtype B, and were either seropositive at entry in the cohort studies (seroprevalent cases with an imputed SC date on average 18 months before entry in the cohort\(^{19,20}\)) or seroconverted during active follow-up in the cohort studies. None of the participants received combination anti-retroviral therapy during the sampling period; samples were obtained on average at 28 months (range 24-33 months) and 51 months (range 45-83 months) after imputed or documented SC.

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 from each participant. The study was approved by the Academic Medical Center institutional medical ethics committee.

**U87/pseudovirus assay for testing of HIV-1 neutralizing activity in serum**

Sera from all 35 patients were tested for neutralizing activity in a pseudovirus assay developed by Monogram Biosciences. The tier 2-3 virus panel that we used for determining cross-neutralizing activity in serum consisted of HIV-1 pseudoviruses from subtypes A (\(n=5\)), B (\(n=6\)), C (\(n=7\)), and D (\(n=5\)) and included recently transmitted isolates, and moderately neutralization sensitive and resistant primary HIV-1 variants, based on previously determined neutralization sensitivities to subtype B sera and MAbs b12, 2G12 and 4E10\(^{21,22}\). Pseudotyped viral particles were produced by cotransfecting HEK293 cells with an expression vector carrying the HIV-1-derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucPCND0-ΔU3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudovirus stocks were then diluted to result in infectivity, as measured by relative light units (RLUs), that fell within a range known to yield reproducible IC\(_{50}\)s.

A recombinant virus assay involving a single round of virus infection was used to measure cross-neutralization activity of the sera\(^{14,23}\). Diluted pseudoviruses were incubated for 1 hour at 37 °C with serial dilutions of serum 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%
Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control. 1:40 was the lowest serum dilution used in the assay. For calculation of IC₅₀ values for viruses that were not inhibited by the 1:40 serum dilution we assumed that 50% inhibition would have occurred at a 1:20 serum dilution.

**PBMC based assay for testing HIV-1 neutralizing activity in serum**

Sera from the 19 individuals with a documented SC were tested in parallel in a PBMC based neutralization assay using both resistant and sensitive tier 2-3 primary HIV-1 variants 92UG029, KNH1144 (subtype A), BX08, BK132 (GS 009) (subtype B), SM145 (GS 016) (subtype C), 92UG038, 93UG065 (subtype D) and CAM1970LE (CRF_AG) 24. PBMCs 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), and phytohemagglutinin (PHA; 5 µg/ml) at a cell concentration of 5 x 10⁶/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, these were inactivated by incubation at 56°C for 30 minutes. From each virus isolate, an inoculum of 20 50% tissue culture infective doses in a total volume of 50 µl was incubated for 1 hour at 37°C with increasing dilutions of the serum (starting concentration 1/25) in 96-well microtiter plates. Subsequently, 10⁵ PHA-stimulated PBMCs were added to the mixtures of virus and serum. After 4 hours of incubation, PBMCs were washed once in 100 µl phosphate-buffered saline after which fresh medium was added. On day 11, virus production in culture supernatants was analyzed in an in-house p24 antigen capture enzyme-linked immunosorbert assay 25. Experiments were performed in triplicate. When possible, 50% inhibitory concentrations (IC₅₀) were determined by linear regression.

**Statistical analysis**

Statistical analyses were performed using the SPSS 16 software package. HIV-1 RNA load in plasma (copies/ml) and CD4+ T cell count in blood (number/ml) at set-point were normally distributed and compared between different groups using independent samples t-test. Titer and breadth of the neutralizing activity in serum were not normally distributed and for estimation of correlation coefficients with either viral RNA load in plasma or CD4+ T cell count in blood at set-point the non-parametric two-tailed Spearman correlation coefficient was used. Ranking for assay correlation was normally distributed and calculated with Pearson correlation. The Kruskall-Wallis test was used to compare neutralization titers.
at both years 2 and 4 post-SC, per virus and between patients who had or lacked cross-reactive neutralizing activity in serum. Geometric means of serum neutralization titers were calculated for each patient per time point and the Mann-Whitney test was performed to compare neutralization titers at both years 2 and 4 post-SC.

RESULTS
Prevalence of HIV-1 specific cross-reactive neutralizing serum activity

We studied 35 participants from the ACS for the breadth of HIV-1 specific cross-reactive neutralizing activity in sera that were obtained on average 28 months (range 24-33 months) and 51 months (range 45-83) after SC. HIV-1 specific cross-reactive neutralizing activity was measured in a cell-based infectivity assay using recombinant viruses that carry a luciferase reporter gene and that are pseudotyped with envelope proteins from tier 2-3 HIV-1 subtype A, B, C, and D. To monitor neutralizing activity not mediated by HIV-1 Env-specific antibodies, each plasma sample was also tested against a recombinant virus stock that was pseudotyped with amphotropic murine leukemia virus envelope proteins (gp70SU and p15TM). Typically, neutralization titers, expressed as the reciprocal of the plasma dilution that inhibited infection by 50% (IC$_{50}$), were <40 for amphotropic murine leukemia virus controls. At 24 months post SC, HIV-specific cross-reactive neutralizing activity, defined as an IC$_{50}$ ≥ 100 for at least 50% of viruses per subtype, from at least 3 different subtypes, was observed in sera from 7 individuals (20%, 3 LTNP and 4 progressors, patient IDs indicated in dark gray in Figure 3.1). At the 4 year time point, sera from these 7 individuals still had high titer cross-reactive neutralizing activity. Interestingly, at this second time point, high titer cross reactive neutralizing serum activity had developed in 4 additional individuals (3 LTNP and 1 progressor, patient IDs indicated in light gray in Figure 3.1) resulting in a prevalence of HIV-1 specific cross-neutralizing serum activity of 31% around 4 years post-SC. There was no difference in prevalence of high titer cross-reactive neutralizing activity in serum between LTNP and typical progressors at either time point of analysis (at year 2 post-SC: 15% of LTNP and 27% of progressors; at year 4 post-SC: 30% of LTNP and 33% of progressors). Moreover, there were no differences in neutralization titers between LTNP and progressors.

Since discrepancies may exist between different neutralization assays, we wanted to confirm our observations obtained with the U87/pseudovirus based neutralization assay in a PBMC based neutralization assay. As the primary viruses from which the pseudoviruses were derived were not available, we used a different panel of 8 primary HIV-1 variants from different subtypes (A, B, C, D and CRF_AG) and serum samples that were obtained 2 years post-SC from 19 ACS participants in this study with a documented SC.

The overall pattern of neutralization of the 8 viruses by the sera from the 19 patients in the BPMC assay is shown in Figures 3.2A and 3.2B, and IC$_{50}$ ≥ 1:100 are indicated in gray. In accordance with our observations in the U87/pseudovirus based assay, the serum
Neutralizing activity in serum as measured in a pseudovirus assay

<table>
<thead>
<tr>
<th>Progressors Long-term non-progressors</th>
<th>Tier 2-3 panel</th>
<th>Reference panel</th>
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<td>19552</td>
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**Figure 3.1:** Breadth and potency of HIV-1 specific neutralizing activity in sera obtained at 2 and 4 years post-SC in progressors (A) and LTNP (B)

IC_{50} values, given as the reciprocal serum dilution of serum samples obtained at 2 years and 4 years post-SC are shown per patient (patients IDs are in the left column). The IDs of patients with cross-reactive neutralizing activity at both 2 and 4 years post-SC are in dark gray, the IDs of patients who developed cross-reactive neutralizing activity in serum between years 2 and 4 are indicated in light gray, IDs of patients who lacked cross-reactive neutralizing activity in serum are indicated in white. In the top row a description of the virus panel is given; the tier 2-3 virus panel consisted of primary subtype A, B, C and D viruses. The references panel (right part) included strains 1196, Bal, JRCSF, NL4-3, and SF162. As a negative control (NC), the amphotropic murine leukemia virus was used. IC_{50} titers ≥1:100 are indicated in gray. Patients are ranked based on the neutralization breadth and potency (most potent serum on the top, least potent serum on the bottom). IC_{50} <40 are indicated with a stripe. n.d., not done.
neutralizing activity against subtype B viruses was the strongest. Unlike the U87/pseudovirus assay, where subtype C viruses were sensitive to serum neutralization, the selected subtype C virus was resistant to neutralization by all but one of the patient sera. Overall, neutralizing serum titers in the PBMC based assay were generally lower, reducing the sensitivity to detect the neutralization breadth of the patient sera as compared to the PV assay. Indeed, in the PBMC based assay, none of the sera were able to neutralize all HIV-1 variants from all different subtypes. However, a significant correlation between the two assays could be observed when patients were ranked based on neutralization breadth and potency (Figure 3.2C). Ranking was assigned by giving priority to serum ability to neutralize different subtypes, followed by the total amount of viruses neutralized and finally by the titers at which the viruses were neutralized.

Figure 3.2: Inter-assay correlation of neutralization by patient sera

Breadth and titer of HIV-1 neutralization in sera from 19 participants of the Amsterdam cohort studies who have a documented moment of seroconversion. The IC_{50} values for the sera that were obtained 2 years post-SC from (A) progressors (n=14) and (B) LTNP (n=5). Patient IDs are shown in the top row. The sera were tested against a panel of subtype A, B, C, D and CRF_AG viruses as shown in the most left column. IC_{50} titer ≥1:100 are color-coded gray. n.d., not done. * 1; primary virus, 2; AIDS repository virus, 3; in PBMC expanded virus. (C) Correlation between breadth and titer of neutralizing activity in patient sera as observed in a PBMC based assay and a U87/PV based assay. Sera were ranked on the basis of neutralization breadth and potency (most broad and highest titer ranked 1, least broad and lowest titer ranked 19). Each dot represents one patient serum and the solid line is the regression line.
Correlation between set-point viral load and CD4+ T cell count and breadth of HIV-1 specific cross-reactive neutralizing serum activity

Our data indicate that cross-reactive neutralizing serum activity does not develop similarly in the course of infection for each HIV-1 infected individual. To obtain some insight into factors that may influence the humoral immune response, we divided the cohort of 35 patients who participated in our study in three distinct groups: patients who had no detectable cross-reactive neutralizing activity at years 2 and 4 post-SC (group A, n=24; patient IDs in white in Figure 3.1), patients who had cross-reactive neutralizing activity already at year 2 post-SC (group B, n=7; patient IDs in dark gray in Figure 3.1), and patients who had developed cross-reactive neutralizing between years 2 and 4 post-SC (group C, n=4; patient IDs in light gray in Figure 3.1). These three groups were compared for plasma viral load and CD4+ T cell count at set-point (Figure 3.3A and 3.3B).

Interestingly, the presence of cross-reactive neutralizing activity at year 2 post-SC (group B) was associated with a higher plasma viral RNA load set-point, as compared to the group of patients who had not developed cross-reactive humoral immunity in the first 4 years post-SC (group A). There was no significant difference in viral RNA load set-point between groups A and C, neither between groups B and C (Figure 3.3A). These observations were reinforced by a significantly lower CD4+ T cell count at set-point in patients with cross-reactive neutralizing activity at year 2 post-SC (group B) as compared to the CD4+ T cell count at set-point in the other 2 patient groups (groups A and C; Figure 3.3B).

Interestingly, an analysis which included all 35 individual patients who participated in our study revealed a positive correlation between the plasma viral RNA load at set-point and the number of viruses that were neutralized by the serum sample obtained at year 2 post-SC (Figure 3.3C). We also observed a negative correlation between the CD4+ T cell count at set-point and the number of viruses that were neutralized by the 2 year post-SC serum sample (Figure 3.3D). No such correlations were observed for the serum neutralizing activity at year 4 post-SC (data not shown).

Viral RNA load and CD4+ T cell count at the time of sampling did not differ between the group that developed cross-neutralizing reactivity (groups B and C) and the group that did not develop cross-reactivity within 4 years post-SC (group A; data not shown).

Titers of HIV-1 specific cross-reactive neutralizing activity in serum increase with duration of infection

With increasing time since SC, we observed an increase in the geometric mean of the neutralizing titers in serum. This could not be explained by the increasing number of patients who developed cross-reactive neutralizing activity over time. Indeed, when we analyzed the 3 groups as defined above (individuals who did not develop cross-reactive neutralizing activity in the first 4 years post-SC (group A), individuals with cross-reactive neutralizing serum activity at year 2 post-SC (group B), and individuals who developed cross-reactive
neutralizing serum activity between year 2 and year 4 post-SC (group C)), the increase in the geometric mean of HIV-1 neutralizing titers in serum was observed in each patient group (Figure 3.4). Even for sera from individuals who did not develop cross-reactive neutralizing activity in the first 4 years post-SC (group A), an increase of the geometric mean of the neutralizing titers was observed over time, albeit that the magnitude of the increase was less than that observed for the patients who did develop cross-reactive neutralizing activity. Finally, we observed that in 91% of patients, an increase in geometric mean of neutralizing titers in serum was observed between years 2 and 4 post-SC.
The geometric mean of IC₅₀ titers of each serum sample for all tier 2-3 viruses in the panel was determined. For each group, defined as having no cross-reactive neutralizing activity (group A), or having cross-reactive neutralizing activity at year 2 post-SC (group B), or year 4 post-SC (group C), the geometric means of IC₅₀ values of serum samples obtained at 2 and 4 years post-SC were plotted. Significant differences are indicated by their p-values.

**DISCUSSION**

We compared 20 LTNP and 15 progressors for the presence of HIV-1 specific cross-reactive neutralizing activity in serum at years 2 and 4 post-SC. Already at 2 years post-SC, 7 individuals (3 LTNP and 4 progressors; overall 20%) had potent cross-reactive neutralizing activity in their sera, defined as the ability to neutralize at least 50% of HIV-1 variants per subtype, from 3 different subtypes, with an IC₅₀ at a serum dilution of >1:100. Interestingly, these 7 individuals had a significantly higher set-point viral RNA load in plasma and a lower CD4+ cell count at set-point than individuals who lacked a potent cross-reactive neutralizing response. The development of potently neutralizing humoral immunity apparently requires exposure to a sufficient amount of antigen, in line with previous observations. Alternatively, a better exposure of epitopes on envelope that are essential for eliciting a cross-reactive neutralizing humoral immune response may coincide with enhanced replication kinetics resulting in a higher plasma viral RNA load set-point.

In a model for Lymphocytic Choriomeningitis Virus (LCMV) infection, a reduction in CD4+ T cell numbers prior to infection reduced polyclonal B cell stimulation and enhanced protective antibody responses in terms of earlier onset and higher titers without impairing protective CD8+ T cell responses. Although the number of patients in our study is low, our observation that early cross-reactive neutralizing activity correlated with a low CD4+ T cell count at set-point may imply that this could also be the case in HIV-1 infection.

The fact that the majority of primary HIV-1 variants are neutralized by one or more of the currently available broadly neutralizing antibodies b12, 2G12, 2F5, and 4E10, already implies that the epitopes for these broadly neutralizing antibodies are accessible on primary
viruses. It is generally assumed however that the configuration of the envelope prevents the elicitation of a neutralizing antibody response \textit{in vivo}. The relatively high prevalence of cross-reactive neutralizing serum activity, which is similar to observations in other studies, suggests however that the relevant epitopes capable of eliciting these humoral responses are accessible and immunogenic on the native gp160 spike of HIV-1, at least in a significant proportion of HIV-1 infected individuals. We may be more conclusive on this point when the exact nature of the neutralizing activity in our study group has been established. Indeed, it is unclear if the breadth of the neutralizing activity in serum is determined by a single high affinity antibody directed against a highly conserved epitope in the envelope protein, or whether cross-reactive neutralizing activity in serum can be attributed to a combination of multiple co-existing neutralizing antibodies directed at a number of distinct regions of the envelope that together give the phenotype of a cross-reactive serum neutralization. It cannot be excluded that both scenarios exist and that it may vary between individuals.

Interestingly, a recent study by Scheid \textit{et al.} has demonstrated the presence of a relatively large memory B cell repertoire capable of producing different antibody specificities in HIV-1 infected individuals with cross-reactive neutralizing serum activity. Irrespective of the nature of the neutralizing response, the 2 to 4 years that seem to be required to achieve a potent neutralizing immune response and then only in 31% of patients, at least in our study population, may hamper the efficacy of vaccine induced humoral immunity. The use of optimal adjuvants may be essential to accelerate the development of broadly neutralizing antibodies after immunization. However, several studies have suggested that low levels of neutralizing titers may actually be sufficient to achieve protection from infection. These lower titers may be achieved more rapidly than the 1:100 serum dilution threshold we set for our experiments shown here.

It remains to be established how HIV-1 neutralizing activity \textit{in vitro} relates to protection from infection \textit{in vivo} which may be better reflected in a PBMC based neutralization assay. However, our initial data on the cross-reactive neutralizing activity in sera obtained with a pseudovirus based assay on U87 cells were confirmed in a PBMC-based assay using replication competent primary HIV-1 variants from different subtypes. Simek \textit{et al.} have demonstrated that neutralizing activity can indeed be reliably assessed using pseudovirus panels. The ability of our patient sera to neutralize viruses from the large virus panel that we used in our present study was strongly correlated with the ability of these same sera to neutralize viruses from the 7 virus panel used by Simek \textit{et al.} (Spearman $r = 0.77$, data not shown) not only confirming the validity of the large virus panel that we used, but also strengthening our conclusion on the broadly neutralizing ability of the patient sera tested in our study.

Although the number of patients in our study is relatively small, our data suggest that there is no correlation between the presence of cross-reactive neutralizing activity in serum and the clinical course of infection. Indeed, we observed a similar prevalence of cross-
reactive neutralizing serum activity at 2 and 4 years post SC in LTNP and progressors. Moreover, the presence of cross-reactive neutralizing antibodies in serum did not coincide with a reduction in viral load, in line with the observation that administration of broadly neutralizing antibodies to hu-PBL-SCID mice after inoculation with HIV-1 had no effect on viral load in the animals 33. Our data are supportive for the idea that it is important to achieve vaccine elicited sterilizing immunity that prevents establishment of infection, or a vaccine that can elicit potent cross-reactive neutralizing humoral immunity in combination with effective cellular immunity to delay or prevent disease progression 34. The relatively high proportion of individuals with cross-reactive neutralizing humoral immunity in our present study and other studies 8,11 suggests that the B cell repertoire in humans should indeed be sufficient to respond to a vaccine with potently neutralizing antibodies implying that a protective antibody-based vaccine against HIV-1 may be an obtainable goal.

ACKNOWLEDGMENTS

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, and the University Medical Center Utrecht, are part of The Netherlands HIV Monitoring Foundation and are financially supported by The Netherlands National Institute for Public Health and the Environment. The research leading to these results has received funding from the European Community’s Six Framework Programme Europrise (FP6/2007-2012) under grant number 037611, the European Community’s Seventh Framework Programme NGIN (FP7/2008-2012) under grant agreement n° 201433, The Netherlands Organisation for Scientific research (NWO; grant 918.66.628) and NIH Small Business Innovation Research (SBIR) grant (5R44AI062522) awarded to Monogram Biosciences. The funding organisations had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: 92UG029, KNH1144, CAM1970LE, BX08 from Dr. Victoria Polonis, BK132 (GS 009), SM145 (GS 016) from Dr. Nelson Michael, 92UG038, and 93UG065.

REFERENCES


Cross-reactive neutralizing humoral immunity does not protect from HIV-1 disease progression
Abstract

Broadly-reactive neutralizing antibodies are the focus of HIV-1 vaccine design. However, only little is known about their role in AIDS pathogenesis and the factors associated with their development. Here we used a multi-subtype panel of 23 HIV-1 variants to determine the prevalence of cross-reactive neutralizing activity in sera obtained ~35 months post-seroconversion from 82 HIV-1 subtype B infected participants from the Amsterdam Cohort Studies. Of these patients, respectively 33%, 48%, and 20% had strong, moderate, or absent cross-reactive neutralizing activity in serum. Viral RNA load at set-point and AIDS free survival were similar for the three patient groups. However, higher cross-reactive neutralizing activity was significantly associated with lower CD4+ T cell counts before and early after infection.

Our findings underscore the importance of vaccine elicited immunity to protect from infection. The association between CD4+ T cell counts and neutralizing humoral immunity may provide new clues to achieve this.
INTRODUCTION
In HIV-1 infected individuals, neutralizing antibodies can develop against autologous HIV-1 strains within weeks of infection. In general, antibodies that can neutralize autologous virus variants are strain-specific and lack the ability to neutralize heterologous viruses. Some HIV-1 infected individuals, however, mount a potent neutralizing humoral immune response that has the in vitro ability to neutralize HIV isolates from unrelated subjects. The exact nature of cross-reactive neutralizing activity in serum is unclear and may be the result of a single high affinity antibody directed against a highly conserved epitope in the envelope protein. Alternatively, it may reflect the activity of several neutralizing antibodies that in combination give cross-reactive neutralizing activity. Little is known about the protective properties of broadly cross-reactive neutralizing antibodies in vivo. In non-human primate studies, passive transfer of broadly neutralizing antibodies completely blocked infection by a chimeric simian-human immunodeficiency virus, while in humans, passive transfer of broadly neutralizing antibodies delayed HIV-1 rebound after cessation of antiretroviral therapy.

In our present study, we wished to determine the prevalence of cross-reactive neutralizing humoral immunity in serum among participants of the Amsterdam Cohort Studies on HIV infection and AIDS and whether the presence of HIV-1 specific cross-reactive neutralizing activity in serum was associated with delayed disease progression. In addition, we wanted to reveal factors that were associated with the development of such a potent humoral immune response.

In our cohort, 33% of participants had cross-reactive neutralizing serum activity, but no correlation between the presence of potent humoral immunity and disease course could be revealed. The mounting of a potent and cross-reactive neutralizing immune response was significantly associated with a lower CD4+ T cell count at set-point but not with viral load at set-point. So although potently neutralizing humoral immunity does not seem to influence disease course, our findings may be relevant for the achievement of optimal vaccine responses.

MATERIALS AND METHODS
Study participants
The study population consisted of 131 Caucasian, homosexual men who were HIV-1 negative at the moment of enrollment between October 1984 and March 1986 in the Amsterdam Cohort studies on the natural history of HIV-1 infection, and who seroconverted for HIV-1 antibodies between 1984 and 1996 during active follow-up. To obtain the best figure on prevalence of cross-reactive neutralizing activity in serum, which generally develops relatively late after seroconversion, we chose serum samples that were obtained at a mean of 35 months (range 30-37 months) after seroconversion. Individuals who had already reached a CD4+ T cell count of less than 200 cells/µl blood, had developed AIDS, had
initiated highly active antiviral therapy or were lost to follow-up at the time of screening were excluded, leaving 82 individuals for analysis.

For Kaplan–Meier survival analysis, individuals were censored at their first day of effective antiretroviral therapy or when lost to follow-up. When AIDS (CDC definition 1993) was used as an end point in Kaplan–Meier survival analysis, 46 individuals had an event, 13 were censored due to loss to follow-up, and 23 were censored because of initiation of highly active antiretroviral therapy (HAART). When AIDS-related death, defined as death with AIDS-related malignancy, death with AIDS-opportunistic infections, or death with AIDS-related cause not specified by the treating physician was used as an end point, 29 individuals had an event, 16 were censored due to loss to follow-up, and 37 were censored at initiation of HAART. For survival analysis after AIDS diagnosis 25 had an event, 20 were censored due to loss to follow-up, and 37 were censored at initiation of HAART.

The Amsterdam cohort studies have been conducted in accordance with the ethical principles set out in the declaration of Helsinki and written informed consent was obtained from each cohort participant prior to data collection. The study was approved by the Amsterdam Medical Center institutional medical ethics committee.

Neutralization assay
Sera from all 82 cohort participants obtained at a mean of 35 months post-seroconversion were tested for cross-reactive neutralizing activity in a pseudovirus assay involving a single round of viral infection as developed by Monogram Biosciences. We used 2 tier 2-3 virus panels (Supplementary Table S4.1) for determining cross-neutralizing activity in serum. The first panel consisted of 20 pseudoviruses with envelope sequences from HIV-1 subtypes A, B, C, and D with 5 viruses per subtype (panel 1). Viruses were obtained recently after transmission or during the chronic phase of infection and were either moderately sensitive or neutralization resistant based on previously determined neutralization sensitivities to sera from subtype B infected individuals and monoclonal antibodies (MAbs) b12, 2G12 and 4E10. The second panel consisted of 5 pseudoviruses with envelope sequences from primary isolates of HIV-1 subtypes A, B, C and CRF01_AE (panel 2) that were resistant (n=1), moderately resistant (n=3) and moderately sensitive (n=1) based on previously determined neutralization sensitivities to sera from subtype B infected individuals and MAbs b12, 2G12 and 4E10. This 5-virus panel covered 93% of the variation in neutralization of a larger pseudovirus panel (n=15) pseudoviruses. Pseudotyped viral particles were produced by cotransfection of HEK293 cells with an expression vector carrying the HIV-1-derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucPCNDO-ΔU3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudovirus stocks were tested and normalized for infectivity prior to testing in the neutralization assay. Diluted pseudoviruses were incubated for 1 hour at 37 °C with
serial dilutions of the patient sera 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% (IC$_{50}$). Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control. 1:40 was the lowest serum dilution used in the assay. For calculation of IC$_{50}$ values for viruses that were not inhibited by the 1:40 serum dilution, we assumed that 50% inhibition would have occurred at a 1:20 serum dilution.

**Viral load measurements**

Viral load in plasma was routinely measured at every study visit in the cohort studies by using a quantitative HIV-1 RNA nucleic acid-based sequence amplification (Organon Teknika, Boxtel, The Netherlands) with electro-chemiluminescently labeled probes. Set point viral load data were available for all patients. Viral load data were analyzed after log 10 transformation.

**Immunologic assays**

CD4$^+$ T cell counts in peripheral blood were first measured at the first visit after entry in the Amsterdam cohort studies, and were routinely measured at every subsequent study visit using flow cytometry. Set-point CD4$^+$ T cell count data were available for all patients. Data on CD4$^+$ T cell and CD8$^+$ T cell percentages from 62 patients were available from a previous study.

**Statistical analyses**

For Kaplan–Meier survival analysis, left truncation was performed for time between seroconversion date and the screening date using S-PLUS 6 (Insightful Corporation, Seattle, Washington, USA). Log rank $P$ value was used to determine differences in the clinical course of infection between groups of patients with either strong, moderate, or absent cross-reactive neutralizing activity in serum. Depending on the distribution of data as determined by the Shapiro-Wilk normality test, the analysis of variance (ANOVA) or Kruskall-Wallis test was used. The association between cross-reactive HIV-1 specific neutralizing activity in serum and viral load at set-point, CD4$^+$ and CD8$^+$ T cell percentages before seroconversion, and 1 and 5 years after seroconversion (normally distributed), were tested with ANOVA. The association between cross-reactive HIV-1 specific neutralizing activity in serum and CD4$^+$ T cell counts at set-point (not normally distributed) was tested with the Kruskall-Wallis test. Spearman’s rank correlation coefficient was used to assess the association of geometric mean titers of each patient serum that were obtained on the two viral panels. Analyses were performed in GraphPad Prism 4 (GraphPad Software, La Jolla, California, USA).
RESULTS

Prevalence of cross-reactive neutralizing activity in serum in the natural course of HIV-1 infection
We first screened sera of participants of the Amsterdam cohort studies on HIV infection and AIDS for the presence of cross-reactive neutralizing activity. As neutralizing serum activity is mounted relatively late after seroconversion, we chose to test sera obtained at 35 months (range 30-37 months) post-seroconversion, which also allowed sufficient follow-up time to perform survival analysis from the moment of screening onwards. Cohort participants who at this time-point had already progressed to disease or initiated HAART were excluded from the study. The remaining group of 82 participants had a median AIDS free follow-up time of 8.31 years (95% CI 5.95 – 10.5 years) after SC when left-truncated for time point of screening. HIV-1-specific cross-reactive neutralizing activity in the sera of these patients was measured in a cell-based infectivity assay using a panel of 20 pseudoviruses carrying a luciferase reporter gene and the envelope proteins (Env) from tier 2 HIV-1 subtype A, B, C, and D (panel 1) and a panel consisting of 6 pseudoviruses with Env from JRCSF and five tier 2 HIV-1 subtype A, B, C, and CRF_01 AE (panel 2) (Supplementary Table S4.1 and Supplementary Figures S4.1 and S4.2).

Cross-neutralizing activity of patient sera on these 2 virus panels (Supplementary Figures S4.1 and S4.2) were strongly correlated (Spearman r = 0.91, Supplementary Figure S4.3). Therefore, data from the two panels were combined for further analysis, excluding the data on CRF_01 AE as we had only 1 variant of this subtype (panel 3; Figure 4.1). The analysis of the combined data sets showed strong correlations between cross-reactive neutralizing activity in serum and geometric mean titer (Spearman r = 0.89, data not shown) and the number of viruses neutralized (Spearman r = 0.79, data not shown). Strong cross-reactive neutralizing activity in serum, defined as the ability to neutralize HIV-1 variants at an IC\textsubscript{50} titer of ≥ 100 to at least one virus from 3 or more subtypes, was observed in 27 patients (33%), similar to the prevalence in other cohorts. Sera from 39 (48%) patients neutralized HIV-1 at an IC\textsubscript{50} titer of ≥ 100 to at least one virus from 1 or 2 subtypes (moderate cross-reactive neutralizing activity) while sera from 16 patients (20%) completely lacked cross-reactive neutralizing activity (Figure 4.1). Interestingly, one patient had an average log transformed neutralizing titer of 2.9 on panel 2, with which he ranked in the top 3 of recently identified elite neutralizers (average log transformed HIV-1 neutralizing titer of >2.5).

Association between cross-reactive neutralizing activity in serum and the clinical course of HIV-1 infection
Next, we investigated the potential relationship between cross-reactive HIV-1 specific neutralizing activity in serum and the rate of HIV-1 disease progression. Kaplan-Meier and Cox Proportional Hazard analysis were performed for the period after the moment at which cross-reactive neutralizing activity in serum was measured, using clinical AIDS (definition CDC 1993) and AIDS-related death as endpoints. In addition, we analyzed the survival
**Figure 4.1: Breadth and titer of HIV-1 specific neutralizing activity in serum**

Shown are the IC50 values given as the reciprocal serum dilution of serum samples obtained at ~3 years post-SC (patient IDs, n = 82, left column). The top row shows virus panel 3 (23 viruses from subtypes A, B, C, and D, and controls on the far right: JRCSF, NL4-3 and amphotropic murine leukemia virus). IC50 < 40 are indicated with a stripe. IC50 titers are color-coded as follows: white, IC50 < 40; light gray, IC50 ≥ 3 times the value of aMLV; gray, IC50 ≥ 1:100; dark gray, IC50 ≥ 1:1000. Patients are ranked based on the neutralization breadth and titer in serum.
time after the moment of AIDS diagnosis, using AIDS-related death as an endpoint. The presence of cross-reactive neutralizing activity in serum was not associated with delayed progression to AIDS according to the 1993 CDC definition (log rank $P = 0.29$; median AIDS free survival times [from the timepoint of screening onwards] for groups of individuals with strong, moderate, or absent cross-reactive neutralizing activity in serum at 35 months post-SC were $7.5 \pm 2.2$, $8.5 \pm 3$, and $10.5 \pm 4$ years, respectively; Figure 4.2A). Time from screening to AIDS-related death was also similar for the groups with strong, moderate, and absent cross-reactive neutralizing activity in serum at 35 months post-SC (log rank $P = 0.69$; median survival times of respectively $9.9 \pm 2.5$, $> 7.9$, and $> 8.5$ years; Figure 4.2B). Finally, survival time after AIDS diagnosis was also the same for the three patient groups (log rank $P = 0.5$; median survival time $2.3 \pm 0.5$ years for individuals with strong cross-reactive neutralizing activity, $2.4 \pm 0.2$ years for individuals with moderately cross-reactive neutralizing serum activity, and $2 \pm 0.4$ years for individuals who lacked cross-reactive neutralizing activity in serum; Figure 4.2C).

**Factors associated with the presence of cross-reactive neutralizing activity in serum**

We subsequently investigated the potential relationship between the breadth of the HIV-1 specific neutralizing activity in serum and the viral RNA load in plasma at set-point and the CD4$^+$ T cell counts at set-point. In our cohort, cross-reactive neutralizing activity in serum at 35 months post-seroconversion was not associated with the level of plasma viremia.

![Figure 4.2: Kaplan–Meier survival analysis from seroconversion till AIDS (CDC 1993) (A), to AIDS-related death (B) and for time from AIDS diagnosis to AIDS-related death (C) for individuals with strong ($n = 27$, red bold lines), moderate ($n = 39$, yellow dashed lines) or absent ($n = 16$ green lines) cross-reactive neutralizing activity respectively. $P$ values (log rank test) are denoted above each figure. Median survival times for groups of individuals with strong, moderate, or absent cross-reactive neutralizing activity at 35 months post-seroconversion were $7.5 \pm 2.2$, $8.5 \pm 3$, and $10.5 \pm 4$ years, respectively for AIDS free survival, $9.9 \pm 2.5$, $> 7.9$, and $> 8.5$ years respectively for median times from seroconversion C to AIDS-related death, and $2.3 \pm 0.5$ years, $2.4 \pm 0.2$ years, and $2 \pm 0.4$ years, respectively for time from AIDS diagnosis to death.](image)
Humoral immunity and HIV-1 control

Interestingly, strong cross-reactive neutralizing activity in serum was significantly associated with a low median CD4+ T cell count at set-point ($P=0.011$; Figure 4.3B). To analyze whether the association between more potent humoral neutralizing activity in serum and CD4+ T cell counts may have a potential significance for vaccine efficacy, we next analyzed whether this same association could be observed between pre-seroconversion CD4+ T cell numbers and the titer of the neutralizing humoral immune response after HIV-1 infection. For this purpose, we compared the mean percentages of CD4+ and CD8+ T cells before seroconversion (at least 6 months before seroconversion) and 1 and 5 years after seroconversion within groups of individuals with strong, intermediate, or absent cross-reactive neutralizing activity in serum. Individuals with strong neutralizing activity had lower percentages of CD4+ T cells ($P=0.011$; Figure 4.3C) and higher percentages of CD8+ T cell counts at set-point, which was defined as the average viral load between month 18 and 24 after seroconversion (Figure 4.3A).

Figure 4.3: Factors associated with the presence of cross-reactive neutralizing activity in serum

Association between strong ($n=27$, ■), moderate ($n=39$, ■), and absent cross-neutralizing activity ($n=16$, □) in serum and (A) set-point log transformed viral RNA load in plasma and (B) CD4+ T cell counts at set-point. (C) Percentage CD8+ T cells or (D) CD4+ T cells before seroconversion and 1 and 5 years after seroconversion within patients with strong (■), moderate (■), and absent cross-reactive neutralizing activity (□) in serum. In Figures C and D, the number of individuals per group ($n$) is denoted below each bar. Mean and standard deviation (A,C,D) or median with interquartile range (B) are shown. $P$-values from the ANOVA (A,C,D) or Kruskall-Wallis test (B) are denoted.
cells ($P = 0.0082$; Figure 4.3D) before seroconversion than HIV-infected individuals who lacked cross-reactive neutralizing activity in serum. This trend was still observed 1 year after seroconversion, but was absent at year 5 of infection (Figures 4.3C and 4.3D).

**Discussion**

Previous studies have shown that autologous strain specific neutralizing activity does not contribute significantly to the control of HIV-1 infection $^{27,29}$. Here we show that even cross-reactive neutralizing activity in serum is not associated with prolonged time to AIDS or death. This observation is in line with the finding that administration of broadly neutralizing antibody b12 before viral challenge could protect animals from infection while administration after inoculation had no effect on the control of established HIV-1 infection in vivo $^{30}$. Moreover, it confirms recent findings in a cohort of Kenyan women in which cross-reactive neutralizing activity was not associated with time to AIDS or initiation of antiviral therapy $^{31}$. Cross-reactive neutralizing activity is known to accumulate with time of infection $^{26}$. For this reason, we chose to screen for serum neutralizing activity at around 35 months post-seroconversion, when an adequate cross-reactive humoral immune response could have been developed, and excluded cohort participants who at that time-point had already developed AIDS, had initiated HAART, or had reached a CD4 count of less than 200 cells/µl blood. As a consequence, individuals with very rapid disease progression were excluded from the analysis and our study design therefore only allows for the conclusion that cross-reactive neutralizing activity has no long-term protective effect on HIV-1 disease progression.

The prevalence of strong cross-reactive neutralizing activity in serum in our study population was 33%, which is similar to observations in recent studies $^{21,25,26}$. Simek *et al.* tested the neutralizing activity in sera from ~1800 individuals on different pseudovirus panels and described that screening on a panel of only 5 selected viruses (panel 2 in our study) provided similar information on the presence of cross-reactive neutralizing activity as screening on a large pseudovirus panel. Indeed, the results obtained with sera from patients in our study on either panel 1, which consisted of 20 viruses from subtypes A, B, C, and D, or panel 2 were highly concordant in geometric mean titer (Spearman $r = 0.91$). This not only confirms the suitability of our large pseudovirus panel for characterization of HIV-1 neutralizing activity in patient sera, it also allows for a direct comparison of our data with previous studies. Interestingly, Simek *et al.* identified 15 so-called elite neutralizers $^{21}$, who had an average log transformed titer that was equal to or greater than 2.5 on panel 2 (including JR-CSF). In our cohort, we identified 1 elite neutralizer who reached a log transformed titer of 2.9 on this same virus panel. When compared to the elite neutralizers in the study by Simek *et al.* $^{21}$, our patient ranked in the 3rd place. Since the prevalence of elite neutralizers is considered to be only 1%, the biomaterial from this Amsterdam Cohort participant is definitely interesting for the identification of potentially novel cross-reactive neutralizing antibodies.
It has been reported that the prevalence of cross-reactive neutralizing activity in serum from elite controllers was much lower as compared to LTNPs and slow progressors. A certain level of antigen is apparently required to drive the humoral immune response. Previous studies have indeed demonstrated a correlation between the breadth of neutralizing activity in serum and viral load at setpoint or at time of testing for neutralizing activity. In our present study, we did not observe a correlation between the presence of cross-reactive neutralizing activity in serum at ~35 months post seroconversion and the viral load at setpoint or at the moment of screening for HIV-1 specific humoral immunity. We currently have no explanation for this apparent discrepancy. However, of the 10 patients with the lowest viral load at setpoint, 6 lacked cross-reactive neutralizing activity in serum, indicating that a certain level of antigen is indeed required to stimulate neutralizing humoral immunity. However, absent cross-reactive neutralizing activity in patients with higher viral load in plasma indicates that additional factors may be critical for the development of a cross-reactive neutralizing antibody response.

We recently demonstrated that in sera of subtype B infected patients, the neutralizing activity was stronger against the subtype B viruses in our panel than against the subtype A, C, and D viruses in our panel. We could confirm this observation in our present study as neutralization of subtype B variants was seen significantly more often than neutralization of viruses from other subtypes (Chi-square P<0.001). Indeed, in sera from 42 out of 82 patients, neutralizing activity against more than 50% of the subtype B viruses in the panel was observed, while neutralization of more than 50% of subtype A, C, or D viruses was seen in sera of only 27, 23, and 19 individuals, respectively.

Interestingly, we observed a correlation between cross-reactive neutralizing activity and a lower CD4+ T cell count at setpoint and a lower CD4+ T cell percentage prior to HIV-1 infection. In another study, this correlation was not seen, but in that study the within-subject average of CD4+ T cells from different time points was compared to neutralization breadth and can therefore not be compared to the CD4+ T cell count at setpoint or prior to HIV-1 infection.

Our data are in line with a study in a lymphocytic choriomeningitis virus (LCMV) mouse model, where either partial CD4+ T cell depletion prior to infection or exclusion of dominant CD4+ T cell epitopes from the vaccine enhanced the generation of NAb responses, due to reduced polyclonal B cell activation. In analogy, decreased CD4+ help may prevent polyclonal B cell activation and hypergammaglobulinemia in HIV infection, favoring the production of neutralizing antibodies. Although the differences in percentages or numbers of CD4+ T cells between groups may be small, it could reflect a critical threshold for proper B cell help.

The absent association between cross-reactive neutralizing immunity and the clinical course of HIV-1 infection is suggestive for rapid viral escape from humoral immune pressure, despite the fact that cross-reactive neutralizing antibodies are considered to be
directed against conserved epitopes. We have indeed observed that HIV-1 can rapidly escape from autologous humoral immunity with cross-reactive neutralizing activity (authors’ unpublished data). Apparently, these escape mutations do not come at a fitness cost to the virus, as has been described for certain escape mutations in conserved epitopes for cytotoxic T lymphocytes (CTL) In agreement, we previously reported that the replication rates of viruses that were resistant to broadly neutralizing antibodies b12, 2G12, 2F5, and/or 4E10 were similar to the replication kinetics of the co-existing neutralization sensitive viruses from the same patient.

In conclusion, cross-reactive neutralizing activity in serum does not seem to have an impact on the clinical course of HIV-1 infection. Possibly, and as observed for other viral infections, CTL rather than neutralizing antibodies may contribute to the control of already established infections while neutralizing antibodies may be essential for protection from infection.

Our data suggest that a broadly neutralizing humoral immune response may be best achieved in the face of reduced CD4+ T cell numbers. Although this may be argued unrealistic to achieve deliberately as part of a vaccination regimen, it could provide clues for achieving better efficacy of an antibody vaccine. Apart from that, the relatively large proportion of individuals with cross-reactive neutralizing humoral immunity elicited by the native HIV-1 envelope may already predict a satisfying response rate once a vaccine will be available.

ACKNOWLEDGEMENTS

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REFERENCES


### Supplementary Table S4.1: Neutralization profiles of pseudoviruses used in the 3 screening panels

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R, Resistant; S, Sensitive; MR, Moderately Resistant; MS, Moderately sensitive; BR, Borderline resistant; VS, Very sensitive; n.t., not tested
shown are the IC50 values given as the reciprocal serum dilution of serum samples obtained at 3 years post-SC in the left column. Patient IDs of the 82 HIV-1 infected individuals are given. The top row shows the virus panel that was used (20 viruses from subtypes A, B, C and D and controls on the far right: ICSR, JRCSF, NL43 and amphotropic murine leukemia virus [AMuLV]). ICs are color-coded as follows: white, IC<1:40; light gray, IC=1:40-1:140; dark gray, IC=1:140. Patients are ranked based on the breadth and titer of the neutralizing activity in serum.

**Figure S4.1: Breadth and titer of HIV-1 specific neutralizing activity in patient sera on a panel of 20 HIV-1 variants from 4 different subtypes (panel 1)**

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Chapter 4

Supplementary Figure S4.2: Breadth and titer of HIV-1 specific neutralizing activity in patient sera on a panel of 5 HIV-1 variants from 4 different subtypes (panel 2) ¹

Shown are the IC₅₀ values given as the reciprocal serum dilution of serum samples obtained at 3 years post-SC tested on a reduced viral panel consisting of 5 viruses (top row) and control virus on the far right (JRCSF, NL4-3, and amphotropic murine leukemia virus [aMLV]). IC₅₀ titers are color-coded as in Supplementary Figure S4.1. Patients are ranked based on the breadth and titer of the neutralizing activity in serum.
Supplementary Figure S4.3: Correlation between breadth and titer of HIV-1 specific neutralizing activity in sera on a panel of 20 (panel 1) and 5 viruses (panel 2) respectively. The geometric mean titer was calculated for all 5 viruses plus lab strain JRSCF. The geometric mean titers of neutralization obtained against the panel of 20 viruses plus JRSCF were compared with the geometric mean titers obtained against the panel of 5 viruses. Spearman r and P-value show correlation between the different panels and are indicated in the graph. Each dot represents the geometric mean titer of one patient on both panels.

REFERENCES
Correlations between HIV-1 subtypes and HIV-1 antibody neutralization sensitivity: significant for vaccine development?
Abstract
The correlates of protection against HIV-1 infection or disease progression are still unknown which causes an immense challenge for HIV-1 vaccine design. Existing effective vaccines against other viruses generate antibodies that either block the initial infection or contribute to the eradication of the virus before it can cause disease. For HIV-1, a protective vaccine capable of eliciting protective neutralizing antibodies does not exist and the difficulties for the generation of such a vaccine are multiple. Conserved elements on the viral envelope glycoprotein, the target of HIV-specific neutralizing antibodies, seem to be poorly immunogenic and attempts to generate an immunogen that can elicit broadly reactive neutralizing antibodies have remained largely without success. In addition, the envelope of HIV-1 is highly variable with respect to amino acid sequence, length of the variable loops, and glycosylation pattern. To cope with the high sequence variation, vaccine-elicited subtype-specific neutralizing antibodies have been suggested as an attractive alternative and recent studies have revealed some evidence for the existence of HIV-1 subtype-specific humoral immune responses. Here, we will review these recent findings and hypothesize on the nature of subtype-specific humoral immunity also in light of their relevance for HIV-1 vaccine development.
INTRODUCTION

More than 25 years after the identification of HIV-1 as the causative agent of AIDS, the correlates of protection against HIV-1 infection are still unknown and researchers continue to debate the most effective vaccine strategies to prevent infection or disease progression. The consensus now is that an effective vaccine should elicit both humoral and cellular immunity. In combination, these responses ideally can protect against acquisition of infection or second best, against disease progression by reducing viral load which will also have an impact on the spread of HIV-1 in the population.

The design of an immunogen that is capable of eliciting neutralizing antibodies is complicated as the recombinant Env protein, even in trimeric form, and vector-expressed HIV-1 envelopes do not seem to expose the relevant epitopes. In addition, vaccine-elicited antibodies will have a tough job as HIV-1 seems to be relatively resistant to neutralizing antibodies. This resistance can be explained from the inaccessibility of relevant epitopes due to the trimeric structure of the HIV-1 envelope protein and the density of glycosylation. Moreover, some epitopes for neutralizing antibodies only emerge after the conformational changes that are elicited by the engagement of the viral envelope glycoprotein with the CD4 receptor, when spatial constraints no longer allow binding of the relatively large immunoglobulins.

Another major obstacle in the development of an effective HIV-1 vaccine is the large sequence diversity, especially of the viral envelope glycoprotein. This high sequence diversity of the HIV-1 envelope glycoprotein is considered to make it more challenging or even completely impossible for a single vaccine to be capable of eliciting a humoral immune response that would cover protection against all possible variants that are being transmitted in a population. Based on phylogenetic analysis, HIV-1 has been categorized into subtypes of viruses that are more similar to each other than to the rest of the viruses, which has fueled the discussion on so-called HIV-1 subtype-specific vaccines. Here, based on our own findings and on those of others, we will discuss whether the high sequence diversity of HIV-1 may indeed be covered by multiple HIV-1 subtype-specific vaccines. We will review the possible correlation between HIV-1 subtypes and neutralization serotypes, and discuss whether subtype-specific HIV-1 vaccines are a relevant option to pursue.

HUMORAL IMMUNE RESPONSE AGAINST HIV-1 IN NATURAL INFECTION

In order to better understand the immunogenicity of the HIV-1 envelope glycoprotein and the host immune response against it, humoral immunity in the natural course of infection has been extensively studied. The majority of HIV-1-infected individuals mount an HIV-1 specific neutralizing humoral immune response within weeks to months after primary infection. This response is considered to be strain-specific as neutralizing activity is generally restricted to the autologous virus variant and mainly directed against the variable regions of the envelope glycoprotein. These antibodies rapidly select for escape variants of HIV-1.
HIV-1 that have become resistant to neutralization as a result of amino acid substitutions, insertions and/or deletions in the variable regions, and/or changes in the glycan shield. Escape from neutralizing antibodies may be mediated by mutations in the epitope as a consequence of which the antibody is no longer able to bind, or by changes in other regions of the envelope that prevent access of the antibody to the neutralizing epitope. Irrespective of the mechanism, such viral escape variants will rapidly be selected by humoral immune pressure and will replace the neutralization sensitive virus variants.

Generally later in the course of infection, neutralizing activity against heterologous HIV-1 variants evolves but only in a much smaller proportion of the HIV-1 infected population. Indeed, the majority of HIV-1 infected individuals do not develop strong cross-reactive neutralizing activity that is capable of neutralizing HIV-1 variants from different subtypes.

**SUBTYPES OF HIV-1 BASED ON GENETIC DIVERSITY**

One of the characteristics of HIV-1 is its enormous sequence diversity. During infection, each day between $10^8$ and $10^{10}$ viral particles are being produced and eliminated. The error-prone viral reverse-transcriptase enzyme and the lack of proofreading mechanisms during reverse transcription of the viral RNA result in frequent mutations in the viral genome. The large turnover of virus in combination with this high mutation rate results in a mixed population of related but distinct HIV-1 variants, also termed the viral quasispecies. Viral variants within a quasispecies are continuously competing, and the dominant sequence reflects the most fit variant at that time point. After accidental introduction of beneficial mutations in the viral genome or due to changing environmental factors, such as the introduction of antiretroviral agents or effective HIV-1 specific immune responses, a previously minor population may become dominant, after which a new, so-called population equilibrium is established.

All viral genes are prone to mutation and the proteins they encode are subject to variation. However, large sequence variation is not allowed in each viral genomic region as this may interfere with viral fitness. For example, the *gag* and *pol* regions are relatively conserved as viruses with mutations in those regions, which generally come at a fitness cost, are outcompeted by coexisting viruses that lack this mutation. Only when the positive selection pressure on such mutations is higher than the fitness cost associated with a mutation, will the mutant virus outcompete the wild type variants.

The envelope glycoprotein of HIV-1 is highly variable, creating an enormous sequence variation which may be as high as 10% within the viral quasispecies in a single individual. Apparently, the regions in which this huge sequence variation occur are not critical to the viral replication process.

Despite the high diversity, some viruses are more closely related to each other which has led to a classification of HIV-1 variants into subtypes (Figure 5.1). The main group (M-group) is
subdivided into subtypes A to K and different circulating recombinant forms (CRFs), which have different geographic distributions. Subtype B for instance predominates in Europe, the Americas, and Australia, whereas subtype C predominates in Sub-Saharan Africa and the Indian subcontinent. The prevalence of intersubtype recombinant strains is increasing and creates even more HIV-1 genetic diversity. The viral envelope glycoprotein currently already differs by up to 35% between subtypes and up to 20% within subtypes, with the variable regions and also the third constant region (C3) being the most diverse between subtypes.

The enormity of the challenge to design a vaccine that can elicit host immunity capable of covering the huge sequence diversity can be put into perspective by comparing it with the influenza virus, where a diversity of less than 2% in amino acid changes can already cause failure in the cross-reactivity of the polyclonal response elicited by the vaccine. Every year a new influenza vaccine has to be developed to protect against the newly emerging serotypes of the influenza virus for the upcoming flu season. In comparison, all different subtypes of HIV-1 are present at the same time in the population, emphasizing the enormous obstacle of genetic variation for HIV-1 vaccine development.

Figure 5.1: Representation of HIV diversity

Neighbor Joining tree generated with all envelope gene sequences of the M-group in the Los Alamos database ($n=1870$) using the GTR distance model with site rate set at gamma shape set at 0.5. Different subtypes and genetic distance bar are indicated.

HIV-1 neutralization serotypes
Evidence for subtype-specific humoral immunity

The high sequence diversity between HIV-1 variants is challenging the possibility of complete coverage of protection against all circulating viruses by vaccine-elicited neutralizing antibodies. In that light, vaccine-elicited subtype-specific neutralizing antibodies may be an interesting alternative to pursue although the existence of HIV-1 neutralization serotypes may be questioned.

Most research has focused on autologous or cross-subtype neutralizing antibodies, while cross-reactive neutralization of different HIV-1 variants within the same subtype has received only little attention. The majority of early studies did not provide evidence for HIV-1 subtype-specific neutralizing activity in serum. However, these studies were performed with only a limited number of HIV-1 variants and sometimes with a pool of patient sera in which different neutralizing epitope specificities may have been mixed. At that time, well characterized virus panels were not yet developed, which also made it difficult to characterize neutralization specificities. Moreover, these studies strongly focused on broadly neutralizing antibodies that by definition neutralize HIV-1 variants from different subtypes. We recently observed that the neutralizing activity in sera of participants from the Amsterdam Cohort studies who are infected with HIV-1 subtype B was preferentially directed against the subtype B HIV-1 variants in a multi-subtype virus panel that also included subtype A, C, and D HIV-1 variants (Figure 5.2). Although not specifically emphasized by the authors, some other recent reports also include data that show that neutralizing activity in patient sera was stronger against viruses that were from the same subtype as the autologous virus that had elicited the neutralizing antibodies in the sera. Simek et al. screened ~1800 sera from individuals from around the world who were infected with HIV-1 of either subtypes A, B, C, D, or recombinant forms, for neutralizing activity against several multi-subtype virus panels. They found that the neutralizing activity in sera indeed tended to be preferentially directed, and with higher potency, against viruses in the panel that were from the same subtype as the virus that had elicited the neutralizing activity, with the strongest correlations for subtypes B and C. These authors also showed that overall, subtype C viruses were neutralized with higher potency than other viruses by heterologous patient plasma, confirming that subtype C viruses are more neutralization sensitive.

The existence of subtype-specific neutralizing antibodies may also have been underestimated in studies that focused on the number of neutralized viruses from different subtypes, while not taking into account the neutralizing titers. With that approach, cross-reactivity will be seen as evidence against subtype-specific neutralizing antibodies while neutralizing titers against viruses from the same subtype as the infecting variant that had elicited these antibodies may have been significantly higher. This would then have pointed to the existence of subtype-specific neutralizing antibodies, or at least neutralizing antibodies with a higher affinity for epitopes that are only present or accessible on viruses from a specific subtype.

Another indication for the existence of subtype-specific humoral immunity is the specificity
Figure 5.2: Heatmap and clustering analysis of neutralizing activity in serum

Sera of 35 patients from the Amsterdam Cohort Studies were tested for neutralization activity against a panel of 23 tier 1 viruses (5 HIV-1 variants from subtype A, 6 from subtype B, 7 from subtype C, and 5 from subtype D). IC$_{50}$ values are shown in gray shade, with the lowest values in white and the highest values in dark gray. Each column shows the IC$_{50}$ values of a single serum, each row shows the neutralization data per virus isolate. The viruses are clustered according to their neutralization sensitivity and the sera are clustered according to their neutralization capacity. The subtype to which the virus belongs is indicated at the end of each cluster branch. In the clustering procedure, the Euclidean distance between the log$_{10}$ IC$_{50}$ values of a set of neutralization values in one row or column was calculated and repeated 10,000 times to find the best fit. Raw data are taken from van Gils et al. 38.
of the neutralizing activity of some of the known broadly neutralizing antibodies. Most broadly neutralizing antibodies have been isolated from subtype B-infected individuals and some of these monoclonal antibodies (mAbs) do not efficiently neutralize non-subtype B HIV-1 variants, either reflected in a lower number of non-subtype B viruses that could be neutralized and/or in lower neutralizing titers against these viruses \(^{26,34,40}\). For example mAb b12 neutralized up to 75% of subtype B viruses, but less than 50% of non-subtype B viruses. Also mAb 2G12 was shown to have a rather limited neutralizing activity against non-subtype B viruses. Although the MPER is relatively conserved, the 2F5 epitope is absent in a large proportion of subtype C viruses, which explains the limited neutralizing activity of the 2F5 antibody against HIV-1 variants of this subtype \(^{12,34}\). MAb 447-52D may be the best example of subtype-specific neutralizing humoral immunity as it completely lacks the ability to neutralize non-subtype B HIV-1 variants but has neutralizing activity against the vast majority of unrelated subtype B viruses, albeit it mainly against highly neutralization sensitive Tier 1 isolates \(^{34}\). Using a heat-map, neutralization data can be organized into meaningful patterns, making it easier to interpret the data. With this tool, Binley \textit{et al.} have shown a clustering of the different subtypes that was based on the neutralization profiles of the broadly neutralizing mAbs mentioned above \(^{34}\).

**Variations in the humoral response elicited by HIV-1 variants from different subtypes**

Because of the high sequence diversity between subtypes and the existence of subtype-specific neutralizing antibodies, it cannot be excluded that the potency and quality of the elicited envelope-specific humoral immune response is associated with the subtype of the HIV-1 variant by which an individual is infected. Epitopes in the first and second variable regions (V1V2) appear to be common immunogens between HIV-1 variants from different subtypes in early infection. However, this region is highly variable and antibodies that target this region are mostly type-specific. The V1V2 region is also highly involved in shielding neutralizing epitopes in other parts of the envelope protein and is frequently associated with the escape from neutralizing antibodies via amino acid substitutions, insertions and deletions, and/or changes in glycosylation \(^{8,9,41-43}\). An additional region that is targeted by the early humoral immune response elicited by different subtypes is the third variable region (V3). Anti-V3 antibodies are among the first antibodies to be elicited in HIV-1 infection and can have cross-subtype neutralizing capacity \(^{44}\). This cross-reactivity appears to be more common for V3-specific antibodies elicited by HIV-1 subtype A infection than for antibodies elicited by subtype B HIV-1 variants \(^{45,46}\), indicating that the epitopes in the V3 domain are indeed different, at least between some subtypes. These differences in V3 epitopes may be used for diagnostic purposes as it turned out to be possible to determine the specific subtype of an infecting strain using a V3 loop peptide immunoassay \(^{47}\). However, anti-V3 antibodies play a minimal role in neutralizing humoral immunity, due to the occlusion of the
V3 loop within the trimeric envelope glycoprotein of primary viruses \(^{44,46,48}\). As mentioned above, HIV-1 subtype C viruses seem to be more sensitive to neutralization than HIV-1 variants from other subtypes \(^{31-33,38}\). Moreover, individuals infected with subtype C HIV-1 variants seem to have a better and stronger neutralizing antibody response than individuals infected with a non-subtype C virus \(^{33,37,49,50}\). Indeed, subtype C HIV-1 infected individuals were most prevalent among elite neutralizers \(^{32}\), confirming the higher immunogenicity of this subtype. It is tempting to speculate that vulnerable epitopes in subtype C viruses are less well occluded by the variable loops and/or the glycan shield of the envelope glycoprotein, making it easier for neutralizing antibodies to access their epitopes and neutralize these viruses. In addition, this enhanced epitope exposure will improve immunogenicity, making it easier to elicit neutralizing antibodies.

One of the major differences between subtype C HIV-1 and viruses from other subtypes is the C3 region in the viral envelope gp120 which for subtype C viruses is a target for neutralizing antibodies \(^{51}\). There are structural differences between subtype B and subtype C HIV-1 variants in the alpha 2-helix of C3 and it has been shown that this region is a target for antibodies \(^{8,48}\). In viruses from other subtypes this region may be much more occluded, interfering with the possibility to elicit neutralizing antibody responses.

Other regions of the envelope glycoprotein, such as the CD4 and co-receptor binding sites and the membrane-proximal (MPER) region of gp41, are much more conserved and very similar between different subtypes. In line with this is the enormous neutralization breadth of antibody 4E10, albeit not very potent, against the MPER region of gp41 and the breadth and very strong potency of the recently described antibody VRC01 that targets the CD4 binding site. The limited breadth of mAb b12, which is also directed against the conserved CD4 binding site, can be explained by the fact that mAb b12 is directed at subtype-specific residues while VRC01 is directed against much more cross-subtype conserved residues in the CD4 binding site \(^{40,52,53}\).

**Characteristics of cross-reactive HIV-1 specific neutralizing activity**

Factors that determine whether broadly neutralizing antibodies develop are largely unknown. A positive correlation between the viral load setpoint in plasma and the breadth of humoral immunity \(^{13,54}\) implicates that at least sufficient antigen exposure is required to elicit potently neutralizing antibodies. Indeed, the prevalence of cross-reactive neutralizing activity is low among elite controllers and long-term non-progressors with low viral load \(^{13,55-57}\). It has also been shown that the breadth of neutralization is correlated with the time since infection \(^{13,15}\). It generally takes almost 2 to 3 years for broadly neutralizing antibodies to develop. This time may be required for the affinity maturation during which the neutralizing antibodies gain affinity and become highly potent, which corresponds with the finding that the breadth of neutralization is also correlated with antibody avidity \(^{13}\). It has been hypothesized that
the development of broadly neutralizing antibodies may also be related to the evolution of HIV-1. As neutralizing antibodies emerge during the course of infection, they will rapidly select HIV-1 escape variants that have mutations in the epitope that is recognized by these antibodies. In turn, these viral escape variants may contribute to the affinity maturation of the neutralizing antibody response. By continuous cycles of selection for escape variants that subsequently drive affinity maturation, antibodies with higher potency and breadth may emerge. There is indeed a strong correlation between the serum titer and breadth of the neutralizing response and both seem to increase simultaneously during the course of infection (Figure 5.3). This may imply that high concentrations of cross-reactive neutralizing antibodies will increase the chance that such an antibody can bind, for instance when the epitope is only transiently accessible in the trimeric structure.

An alternative hypothesis is that instead of affinity maturation of the original antibody response, the constantly emerging escape variants continuously elicit novel antibody responses during the course of infection which in combination may provide a serum with a cross-reactive neutralizing phenotype. Indeed, recent studies have demonstrated that the individual epitope specificities did not account for the breadth of neutralizing activity in serum whereas the combination of these different antibodies did approach the neutralization phenotype of the patient serum.

To be more conclusive on the nature of cross-reactive neutralizing humoral immunity, the neutralizing component in serum needs to be identified. This will show whether the breadth of the neutralizing activity in serum is determined by a single high-affinity antibody directed against a highly conserved epitope in the envelope protein, or whether cross-reactive neutralizing activity in serum can be attributed to a combination of multiple co-existing neutralizing antibodies directed against a number of distinct regions of the envelope that

Figure 5.3: Correlation between serum titer and neutralizing breadth

On the x-axis an increasing heterologous neutralization titer is suggested, on the y-axis an increasing breadth of the response in three categories. Line represents the association between neutralizing titer and breadth, increasing gray tone in the background shows increasing potency of neutralizing activity. Figure is reproduced from van Gils et al. 38.
HIV-1 neutralization serotypes

together give the phenotype of a cross-reactive serum neutralization. It cannot be excluded that both scenarios exist and that the number of antibody specificities in cross-reactive neutralizing sera may vary between individuals.

It is likely that epitopes that are less well conserved between subtypes but conserved within a subtype are capable of eliciting subtype-specific, rather than cross-reactive neutralizing antibodies. The epitope of the already mentioned 446-52D in the V3 loop is an example of this. This epitope is quite different between subtypes but highly conserved in subtype B HIV-1 variants, underscoring that subtype-specific neutralizing antibodies indeed exist.

The potential relevance of subtype-specific neutralizing antibodies as a target in vaccine development remains to be established. The idea to use this type of antibodies stems from the notion that cross-subtype neutralizing antibodies are extremely rare. However, several recent studies have demonstrated that a relatively high proportion (~30%) of individuals has cross-reactive neutralizing humoral immunity, which suggests that the epitopes that are capable of eliciting these humoral responses are accessible and immunogenic on the native gp160 spike of HIV-1 and that the B cell repertoire in humans is indeed capable of producing these potently neutralizing antibodies, at least in a significant proportion of HIV-1 infected individuals. Interestingly, although cross-reactive neutralizing activity is considered to be directed against epitopes that are conserved in HIV-1 variants from different subtypes, also here a rapid selection of escape variants is observed without a major impact on viral fitness. This suggests that escape from cross-reactive neutralizing activity is not mediated by mutations in the conserved epitopes but rather by changes in the variable regions that then prevent access of the neutralizing antibodies to their target epitopes.

The fact that HIV-1 rapidly escapes from even the most potent and cross-reactive neutralizing antibodies implicates that by all means, viral replication in a new host should be prevented. A vaccine therefore should elicit protective immunity that protects against acquisition of HIV-1.

**Directions for vaccine development**

It is generally assumed that an HIV-1 vaccine should elicit humoral immunity, ideally in combination with a cellular immune response. In line with this assumption is the observation that all vaccine formulations that have been tested in phase 2b trials, all lacked an immunogen that was capable of eliciting strongly neutralizing antibodies, which had little or no impact on HIV-1 transmission. In initial trials, subtype B monomeric envelope glycoproteins elicited high titer type-specific antibody responses against the vaccine strain, but did not provide protection against acquisition of HIV-1 in the population. A polyvalent envelope glycoprotein vaccine-elicited neutralizing activity against tier 1 viruses, but still no protection from infection by a heterologous SHIV in macaques was achieved.

A first modest success was obtained with a pox virus prime, gp120 protein boost vaccine regimen in the so called Thai trial (RV144). This vaccine included gag, nef, and pol and in
addition monomeric envelope glycoproteins from subtypes B and E, which are the major circulating subtypes in the region where the vaccine trial was performed. The vaccine-induced protective effect was however only modest and the identification of the immune correlates of protection and the relative contribution of each vaccine component need to be elucidated. First analyses have shown that vaccinated individuals developed HIV-1 binding antibodies in serum but data on neutralizing activity of these antibodies has not yet been reported. It cannot be excluded that other antibody functions, such as ADCC or ADVCI play a role in the achieved protection. Another interesting question to address is whether the HIV-1 variants that established infection in the vaccine recipients are genetically different from the vaccine strains or from the viruses in the infected placebo recipients. The nature of neutralizing antibody responses in natural HIV-1 infection may offer new clues for vaccine design. Recently, the extremely potent and broadly neutralizing antibodies VRC01 and PG9 and PG16 were identified, which all seem to target conserved regions of the envelope glycoprotein. Similar to mAb b12, VRC01 targets the CD4 binding site, but with a much higher potency. PG9 and PG16 recognize a conformational epitope in the V2 region that for its conformation is dependent on glycosylation of the V2 region. In the retrovaccinology approach, these epitopes will serve the design of novel immunogens.

In addition to the approach described above, progress has been made on the characterization of the consensus and/or most recent common ancestor sequences that are considered to resemble HIV-1 variants that have not yet escaped from immunity and that may harbor important epitopes. A new development is the use of a mosaic vaccine to optimize the immunogenicity in an attempt to elicit subtype-specific or even cross-subtype neutralizing antibodies. Mosaic sequences closely resemble natural strains and by design are assembled from common mutational solutions that the virus itself favors to balance fitness constraints with immune escape. B-cell mosaics are being developed to optimize epitope coverage. A cocktail of mosaics in a vaccine might elicit potently neutralizing antibody responses.

**Conclusion**

Subtype-specific humoral immunity has been suggested as an interesting alternative to deal with the huge sequence variation that is challenging HIV-1 vaccine development. As reviewed here, at least one neutralizing antibody is more or less subtype-specific as its epitope is mainly present in viruses that belong to subtype B. The fact that truly broadly neutralizing antibodies that neutralize HIV-1 variants from different subtypes exist, implicates that specific epitopes that can elicit these antibody responses are highly conserved across HIV-1 subtypes. The sequence variation in the HIV-1 envelope may thus be less problematic for the choice of epitope specificities a vaccine should cover. Indeed, it may not so much be a matter of whether an epitope is present but rather if it is accessible on HIV-1 variants from different subtypes.

Novel antibodies such as VRC01, PG9 and PG16, with unmet breadth and potency,
HIV-1 neutralization serotypes should be the starting point for vaccine design as these antibodies, in real life, seem to have overcome the problem of subtype-specificities.

**Acknowledgments**

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**References**


HIV-1 neutralization serotypes
Chapter 6

Genetic composition of replication competent clonal HIV-1 variants isolated from peripheral blood mononuclear cells (PBMC), HIV-1 proviral DNA from PBMC and HIV-1 RNA in serum in the course of HIV-1 infection
**ABSTRACT**

The HIV-1 quasispecies in peripheral blood mononuclear cells (PBMC) is considered to be a mix of actively replicating, latent, and archived viruses and may be genetically distinct from HIV-1 variants in plasma that are considered to be recently produced.

Here we analyzed the genetic relationship between gp160 env sequences from replication competent clonal HIV-1 variants that were isolated from PBMC and from contemporaneous HIV-1 RNA in serum and HIV-1 proviral DNA in PBMC of four longitudinally studied therapy naïve HIV-1 infected individuals.

Replication competent clonal HIV-1 variants, HIV-1 RNA from serum, and HIV-1 proviral DNA from PBMC formed a single virus population at most time points analyzed. However, an under-representation in serum of HIV-1 sequences with predicted CXCR4 usage was sometimes observed implying that the analysis of viral sequences from different sources may provide a more complete assessment of the viral quasispecies in peripheral blood in vivo.
**INTRODUCTION**

During the course of HIV-1 infection virus replication and viral turnover are high which, in combination with the error-prone nature of the HIV-1 reverse transcriptase and its lack of proofreading, contribute to the high genetic variability of HIV-1 and result in a continuous emergence of new HIV-1 variants. The random generation of viral mutants facilitates escape from host immune pressure and antiviral drugs, and selection for biological properties such as co-receptor use and replication capacity.

The half life of HIV-1 in plasma is about 1.3 hours indicating that virions present in this compartment have been produced very recently. The CD4+ T cell subset in peripheral blood mononuclear cells (PBMC) is one of the targets for HIV-1, and, once infected, produces new viral progeny, part of which will be replication competent and likely to contribute to the viral quasispecies found in plasma. Viruses produced in other anatomical compartments may however also contribute to the composition of the viral population in plasma, at least to some extent or in certain disease stages. While plasma is considered to harbor recently produced virus variants, PBMC are considered to harbor a combination of recently produced and archived virus variants as they accumulate integrated viral DNA. Taken together, this would imply that the genetic composition of the viral populations derived from plasma and PBMC may be different.

Whereas sections of the viral genome can be functionally evaluated using recombinant pseudovirus assays, the study of biological properties of full-length replication competent HIV-1 requires virus isolation in vitro. Given that the direct isolation of full-length replication competent viruses from plasma is not very efficient, virus isolation is usually done by coculturing of patient PBMC with stimulated healthy donor PBMC. However, during virus isolation in bulk culture, the patient’s HIV-1 variant that is most fit for replication in PBMC will be rapidly selected and outgrow the less fit virus variants that coexisted in the patient in vivo. To overcome this, we designed a protocol in which limiting numbers of HIV-1 infected patient PBMC and stimulated healthy donor PBMC are mixed in multiple parallel cocultures. This procedure allows for the isolation of multiple replication competent clonal HIV-1 variants (CV) from a single PBMC sample and avoids outgrowth and loss of slowly replicating variants.

To investigate the genetic relationship between viral variants derived from the cell-free (serum) and cell-associated (PBMC) virus pool and to examine whether CV are representative for the replication competent viral quasispecies in peripheral blood, we compared HIV-1 gp160 env sequences from replication competent CV with contemporaneous viral gp160 env sequences derived directly from serum (viral RNA) and PBMC (proviral DNA) in a longitudinal study of 4 combined antiretroviral therapy (cART) naïve HIV-1 infected individuals throughout their course of infection.
MATERIALS AND METHODS

Patients and samples

Longitudinal blood samples from eight participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS (http://www.amsterdamcohortstudies.org) were used for this study. Patients A, B, C and D were selected on the basis of available CV isolated from PBMC at multiple time points in the course of infection, and the availability of serum and DNA samples from most of these same time points. Additionally, patients A and B were selected because they developed CXCR4-using CV in their course of infection while patients C and D were selected because they did not develop CXCR4-using HIV-1 variants. To substantiate our findings on prevalence of X4/CXCR4-using viruses in cell-free and cell-associated HIV-1 populations, four additional patients, E, F, G and H, were selected because they developed CXCR4-using HIV-1 variants during their course of infection and because viral sequences from serum-RNA and CV were available from multiple time points covering their course of HIV-1 infection. Time from documented or imputed seroconversion (SC) at the moment of sampling and number of sequences analyzed are indicated in Supplementary Table S6.1 for each patient. Patients did not receive cART during the study period. For each patient longitudinal data on CD4 counts and viral load are shown in Supplementary Figure S6.1. The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set out in the declaration of Helsinki and written informed consent was obtained prior to data collection. The study was approved by the Academic Medical Center institutional medical ethics committee.

Isolation of replication competent clonal HIV-1 variants

Replication competent clonal HIV-1 variants (CV) were obtained in cocultures of longitudinally sampled patient PBMC and 2- to 3- day phytohemagglutinin (PHA) stimulated PBMC from a healthy donor (PHA-PBMC) as described previously. In brief, PBMC from a healthy donor were stimulated for 2-3 days in Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) supplemented with 10% Fetal Calf Serum (FCS; Hyclone), 1mg/ml PHA (Welcome), 100U/ml Penicillin and 100U/ml Streptomycin (Pen/Strep; Gibco Brl), 5mg/ml Ciprofloxacin (Bayer) in a culture flask at a cell density of 5x10^6/ml. Increasing numbers of patient PBMC (range 2,500-40,000) were added to 1x10^5 PHA-PBMC (48 parallel micro-cocultures per patient PBMC number) in a final volume of 200µl IMDM-IL2 medium (IMDM supplemented with 10% FCS, 100U/ml Penicillin and 100U/ml Streptomycin, 10U/ml rIL-2 (proleukin; Chiron Benelux BV), 5mg/ml Ciprofloxacin and 5mg/ml polybrene (Sigma)) for 28 days in a 96-well flat-bottom microtiter plate. Every week, culture supernatants were tested for virus production in an in-house Gag p24 antigen capture enzyme-linked immunosorbent assay (ELISA). At the same time, half of the remaining resuspended culture volume was transferred to new 96-well plates and fresh PHA-stimulated healthy donor PBMC were added to propagate the culture. If less than
HIV-1 evolution in PBMC and plasma

one-third of the 48 parallel micro-cocultures per patient PBMC number is positive for p24 production, then according to Poisson distribution these cultures can be assumed to be infected with progeny virus of one infected patient cell and, therefore, they are highly likely to be clonal. Cultures of clonal viruses were expanded by cocultivation of PBMC from p24 positive micro-cocultures with 5x10⁶ PHA-PBMC at a density of 1x10⁶/ml IMDM-IL2 medium in a culture flask. From all patients, virus isolations were performed at 4 to 6 time points spanning the course of infection.

**HIV-1 RNA isolation from serum, cDNA synthesis and PCR amplification**

Serum RNA was isolated with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s protocol using 140µl of serum. Isolated RNA was eluted in a final volume of 40µl. Viral RNA (10µl) was amplified in an RT-PCR reaction with a total volume of 50µl using Superscript III One-step RT-PCR with High Fidelity Platinum Taq (Invitrogen) and primers EnvA (fw) (5'-GGCTTAGGACATCTCATGCGAGGAAGGAA-3') and Env3Rlong (rev) (5'-GGTGTGTAGTTCTGCCAATCAGGGAAGWAGCCCTTGTGTG-3'). The RT step was performed at 55˚C for 1 hour, followed by a 2 min denaturation at 95˚C and 40 PCR amplification cycles of 95˚C for 20 sec, annealing for 20 sec, and 68˚C extension for 4 min. Annealing temperatures were 65˚C for 3 cycles, 60˚C for 11 cycles and 55˚C for 26 cycles. A 25 cycle nested PCR reaction was performed on samples that generated insufficient first round product to be visualized by electrophoresis using Advantage 2 Polymerase mix (Clontech) with internal primers Env_2Flong (fw) (5'-GGTTAATTGATAGAATWAGRGAAAGGCAAGAAGACAGTGCGAATG-3') and Nef5 (rev) (5'-CCCWTCCAGTCCTTTTCTTTAAGAAG-3'), annealing at 55˚C. Gp160 env PCR products were gel purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol.

**DNA isolation from patient PBMC and PCR amplification of proviral DNA**

Total DNA was isolated from 3x10⁶ PBMC from HIV-1 infected patients using a modification of the L6 isolation method. Precipitated DNA was dissolved in 100µl of distilled water and 5µl were used for gp160 env gene amplification with the Advantage 2 Polymerase Mix (Clontech) and primers targeting env (EnvA and Env_3Rlong) in a total reaction volume of 50µl. Reactions were as described for the RT-PCR with omission of the 55˚C RT step. A 25 cycle nested PCR reaction was performed with internal primers Env_2Flong and Nef5 on samples that generated insufficient first round product to be visualized by electrophoresis as described in the previous section. The PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol.

**Cloning and sequencing of gp160 env PCR products from serum and PBMC**

Purified PCR products were cloned into the pCR®4-TOPO vector from the TOPO
Chapter 6

TA Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer’s protocol. Following transformation into One-shot TOP10 chemically competent *E. coli*, positive transformants were selected on LB plates with ampicillin. Eight colonies per sample were picked and shipped to Functional Biosciences, Inc, Madison, WI, USA for sequencing. Plasmid DNA was extracted and sequences were analyzed on an Applied Biosystems 3730xl Genetic Analyzer. Primers used for sequencing are specified in the Supplementary Methods section. Viral load at the time of sampling did not correlate with the diversity of the sequences obtained from serum-RNA or PBMC-DNA, excluding template resampling as a reason for low diversity in some samples.

DNA isolation, PCR amplification and sequencing of gp160 env from clonal HIV-1 variants

Total DNA was isolated from 1x10⁶ healthy donor PBMC in vitro infected with clonal HIV-1 isolates, using a modification of the L6 isolation method and gp160 env gene amplification was performed with one outer PCR with primers TB3 (fw) (5’-GGCCTTATTAG-GACACATAGTAGCC-3’) and TBC (rev) (5’-GCTGCTTTGTAAGTCATTGGGTTCT-TAAAGG-3’) and a nested PCR with primers TB2 (fw) (5’-CTCTACAATACCTTG-GCAGTCAGCCAGGCACA-3’) and TBA (rev) (5’-CTCTTTGCTTGTAGCAGGCACA-3’) using the expand high fidelity Taq polymerase kit (Roche) and the following amplification cycles: 2 min 30s 94˚C, 9 cycles of 15s 94˚C, 45s 53˚C, 6 min 68˚C, 30 cycles of 15s 94˚C, 45s 58˚C, 6 min 68˚C, followed by a 10 min extension at 68˚C and subsequent cooling to 4˚C. PCR products were purified using ExoSAP-IT (USB) according to the manufacturer’s protocol. Sequencing conditions were 5’at 94˚C, 30 cycles of 15” at 94˚C, 10” at 50˚C, 2’ at 60˚C and a 10’ extension at 60˚C. Sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing kit (ABI Prism, Applied Biosystems) according to the manufacturer’s protocol. Primers used for sequencing are specified in the Supplementary Methods section. Sequences were analyzed on the 3130 xl Genetic Analyzer (Applied Biosystems).

Prediction and determination of co-receptor usage

All study participants were routinely tested at approximately 3-monthly intervals for the presence of replication competent CXCR4-using HIV-1 variants in PBMC using the MT-2 assay. In brief, 1x10⁶ patient’s PBMC were cocultured with 1.6x10⁶ MT-2 cells during 3-4 weeks in duplicate and cultures were periodically screened for syncytia formation and for p24 antigen production in the culture supernatant. The midpoint between the last negative and first positive MT-2 test was estimated as the date of first emergence of CXCR4-using viruses in the patient.

All isolated clonal HIV-1 variants were tested for their ability to use CXCR4 in the MT-2 assay. For patient E, CXCR4-using variants were isolated via clonal virus isolation whilst the MT-2 test performed on the same PBMC sample was still negative at that time point, which is most likely related to the lower number of cells that is used for the MT-2 assay.
HIV-1 evolution in PBMC and plasma

(1x10^6 patient PBMC) as compared to the clonal viral isolation procedure (up to 5x10^6 patients PBMC). Indeed, the relative contribution of CXCR4 using HIV-1 to the total viral load may initially be low. CXCR4-using phenotype of CV was confirmed with V3 amino acid sequences and the PSSM matrix \(^{24,25}\) and using the geno2pheno\_[coreceptor] method (FPR = 5\%). \(^{26}\) The same prediction methods were used to predict co-receptor use of viral sequences obtained from viral RNA in serum or from proviral DNA in patient PBMC. Throughout the manuscript we use the term “CXCR4-using” for CV for which actual CXCR4 usage has been demonstrated in the MT2 cell-line, not excluding the possibility that these CV also have the ability to use CCR5 or any other coreceptor. For sequences with a predicted ability to use CXCR4 we use the term “X4” since actual usage of this coreceptor has not been experimentally proven. The term X4 does not exclude the possibility that the envelope has the ability to also use CCR5 or any other coreceptor. The term “CCR5-using” is used for CV which failed to infect the MT2 cell line and the term “R5” is used for sequences with a predicted ability to use CCR5.

**Phylogenetic analysis**

Nucleotide sequences were aligned using ClustalW included in the software package of BioEdit v.7.0.9 \(^{27}\) (BioEdit v 7.0.5, Tom Hall, Ibis Therapeutics, Carlsbad, CA) and edited manually.

Neighbor-Joining (NJ) tree \(^{28}\) for the gp160 envelope sequences from serum, proviral DNA from patient PBMC and isolated clonal HIV-1 variants from the 4 patients under study and a reference sequence panel from different HIV-1 subtypes obtained from Los Alamos Database was constructed under the Hasegawa-Kishino-Yano (HKY85) model of evolution \(^{29}\) in PAUP* 4.0 beta 8 software package \(^{30}\) (http://paup.csit.fsu.edu/). Phylogenetic confidence was assessed by bootstrap with 1000 replicates (data not shown).

The best-fit nucleotide substitution model for every patient sequence data set was selected by hierarchical likelihood ratio test (hLTR) in Model Test 3.7 \(^{31}\) and implemented in the construction of maximum likelihood (ML) trees for the gp160 env region per patient. The heuristic search for the best tree was performed using a NJ tree as starting tree and the TBR branch-swapping algorithm. NJ trees were constructed under the HKY85 model with a transition/transversion ratio and the shape of the \(\gamma\)-distribution estimated using maximum likelihood. ML trees were rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time.

**Analysis of genetic distance**

To estimate diversity and divergence, pairwise nucleotide distances were calculated with the Kimura-2 parameter model of evolution in the software package MEGA 4 \(^{32}\). Mean pairwise distances were compared using the Mann-Whitney test for independent samples (SPSS 16.0 software package).
Statistical tests for compartmentalization

Six different compartmentalization tests were used to determine compartmentalization between sequences from viral RNA in serum, from proviral DNA in PBMC, and from clonal HIV-1 variants. Four of these tests are based on the tree topology (Slatkin-Maddison (SM) 33, Simmonds Association Index (AI) 34 and Correlation Coefficients (r and r_b) 35) and two of them are based on pairwise genetic distances between viral sequences (Wright’s measure of population subdivision (FST) 36-38 and Nearest-neighbor statistic (Snn) 39). Compartmentalization between sequences derived from the three sources was analyzed with four tests (SM, AI, r and r_b). In addition, a pairwise comparison of sequences from two sources was performed with all six tests. Analyses were implemented in ML trees or alignments that included only the gp160 env sequences from time points at which the sources to be compared were available. ML trees were constructed as described above. Statistics and compartmentalization tests were implemented in HyPhy as described 40. Methods and parameters implemented in Hyphy for each method are described in Supplementary Material and Methods.

Results

Phylogenetic analysis of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants isolated from PBMC

Neighbor-joining phylogenetic tree analysis of gp160 env nucleotide sequences from all four patients and a reference sequence panel from different HIV-1 subtypes showed monophyletic clustering per patient of sequences derived from viral RNA in serum (serum-RNA), proviral DNA in PBMC (PBMC-DNA) and replication competent PBMC-derived clonal HIV-1 variants (CV) with high bootstrap support (values between 80 and 100), indicating absence of cross-contamination between patient samples (data not shown).

To better examine the intra-host genetic relationship between the HIV-1 quasispecies in serum-RNA, PBMC-DNA, and CV, maximum likelihood (ML) trees were constructed with gp160 env sequences from each patient separately. Tree topologies revealed temporal structure in all four patients: sequences derived from serum-RNA, PBMC-DNA or CV that had been sampled at the same time tended to cluster together, and sequences from samples collected later in infection showed greater divergence from the root of the tree. The degree of intermingling of sequences from the three different sources varied between patients and per time point. For patient A (Figure 6.1), we observed separate clustering of PBMC-DNA sequences at the first and third time point, respectively, but intermingling of the PBMC-DNA sequences, mainly with CV sequences, at later time points. Sequences from serum-RNA and CV from the first four time points intermingled, but later serum-RNA sequences clustered separately from both contemporary PBMC-DNA and CV sequences, especially at the last time point where they formed a well supported monophyletic cluster that considerably diverged from the other sequences. In patient B (Figure 6.2), PBMC-DNA
sequences collected at the first and second time point intermixed with CV sequences. At the third and fourth time point, viral sequences from all three sources were interspersed. The tree also showed that the majority of serum-RNA sequences from the first two time points and two PBMC-DNA sequences from the third time point formed separate phylogenetic lineages. Only two serum-RNA sequences from the first and second time point, respectively, clustered with sequences from later serum-RNA samples, and with all the CV and the majority of PBMC-DNA sequences. All serum samples were processed separately from the PBMC samples and the CV, excluding contamination between samples from the three different sources and implying that the two clusters of viral env sequences from early serum-RNA samples and the two PBMC-DNA sequences from the third time point of patient B had indeed evolved independently from the other sequences of this patient. In patient C and D (Figures 6.3 and 6.4), intermingling of sequences from the three different sources could be observed at all time points. However, similar to the observation for patient B, five sequences from serum-RNA of the first three time points of patient C clustered separately from the remainder of the sequences of that patient.

Figure 6.1: Maximum-likelihood tree of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants.

ML tree was rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time. Bootstrap support with value >70% is shown. * indicate archived sequences. The scale bar (horizontal line) indicates branch length corresponding to 0.01 substitutions per site.
Figure 6.2: Maximum-likelihood tree of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants

ML tree was rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time. Bootstrap support with value >70% is shown. * indicate archived sequences. The scale bar (horizontal line) indicates branch length corresponding to 0.01 substitutions per site.
Patient C

1: 34 months after SC
2: 48 months after SC
3: 72 months after SC
4: 87 months after SC

Figure 6.3: Maximum-likelihood tree of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants
ML tree was rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time. Bootstrap support with value >70% is shown. * indicate archived sequences. The scale bar (horizontal line) indicates branch length corresponding to 0.01 substitutions per site.
Figure 6.4: Maximum-likelihood tree of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants

ML tree was rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time. Bootstrap support with value >70% is shown. * indicate archived sequences. The scale bar (horizontal line) indicates branch length corresponding to 0.01 substitutions per site.
CXCR4-using variants, which were detected in patients A and B, formed a monophyletic cluster irrespective of the time of sampling. Interestingly, sequences with an X4 signature were only obtained from PBMC-DNA and CV.

Few sequences per patient were identified as archived (marked with * in the phylogenetic tree) as they clustered with sequences from earlier time points and had diverged less from the root of the tree than other sequences from the same time point. We found a total of 2 archived sequences in serum-RNA (in patients C and D), 4 in PBMC-DNA (1 in patient A and 3 in patient C) and 6 in CV (2 in patient B and 4 in patient D).

**Gene flow between sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants**

Compartmentalization tests are generally used to detect a restriction of gene flow between viral subpopulations, in which case each virus subpopulation, generally coming from different anatomical compartments, can evolve independently and become genetically distinct. Here, we used these tests to determine whether there was evidence for frequent gene trafficking between virus variants derived from serum-RNA, PBMC-DNA and CV as if they belonged to a single virus population, or whether gene trafficking was restricted implying differences in genetic composition and independent evolution between virus variants of the different sources.

ML trees and alignments based on envelope sequences of each patient were analyzed with six different compartmentalization tests: SM, r, r_b and A1 that are all based on tree topology, and F_St and Snn that are based on pairwise genetic distances between viral sequences. The 4 tree-based tests, which allow for simultaneous comparison of more than two sequence populations, were used for the analysis of gene trafficking between serum-RNA, PBMC-DNA and CV. In addition, a pairwise comparison of two sources (serum-RNA vs CV, CV vs PBMC-DNA and serum-RNA vs PBMC-DNA) was performed with all six tests (Table 6.1). Comparisons included only the time points at which sequences from all the sources compared were available. Given the different sensitivities and frequently discordant predictions of the different methods, for each comparison we took a majority consensus approach meaning that the signal for compartmentalization was only considered positive when more than half of the tests gave significant P-values.

For patients A, C and D, borderline significant compartmentalization was detected in the simultaneous analysis of all three sources of viral sequences (2 of 4 tests with a significant P-value) suggesting that the viruses from serum-RNA, PBMC-DNA and CV in these patients are likely to form a single virus population. This was supported by the comparisons of each pair of sequence sources separately in which ≤3 of 6 tests gave significant P-value.

For patients A and C, we obtained a borderline significant result (3 of 6 tests positive for compartmentalization) for the compartmentalization analysis between PBMC-DNA and either serum-RNA or CV. This coincided with a certain degree of segregation, at some time
points, between PBMC-DNA and either serum-RNA or CV sequences in the phylogenetic tree of those two patients. For patient B, significant \( P \)-values were obtained in all 4 compartmentalization tests in which sequences from serum-RNA, PBMC-DNA, and CV were analyzed together. Analysis of gene flow for each pair of sequence sources separately showed that compartmentalization occurred between serum-RNA and either PBMC-DNA or CV (\( \geq 5 \) of 6 tests with a significant \( P \)-value). This was supported by the tree topology in which only two serum sequences from the first and second time points clustered with the sequences from later serum-RNA samples and all the CV and the majority of PBMC-DNA sequences, while all other serum-RNA sequences from the first and second time point and two PBMC-DNA sequences from the third time point formed a separate cluster.

Table 6.1: Results of compartmentalization tests per patient and time point

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Compartmentalization analysis was performed with 4 tests (SM, AI, \( r \) and \( r_b \)) when sequences of all three sources (Serum-RNA (SE); clonal HIV-1 variants (CV); PBMC-DNA (PB)) were compared (ALL). Subsequently, all 6 tests were implemented on the comparison of each pair of sources separately (SE vs CV, CV vs PB and SE vs PB) including only the time points at which sequences from the two sources compared were available. The signal for compartmentalization was only considered positive when more than half of the tests gave a significant \( P \)-value (filled cells). \( P \)-values < 0.05 were considerate significant. ns: not significant.

SM: Slatkin-Madison; AI: Simmonds Association Index; \( r \): distance between to sequences as the number of tree branches separating them in the phylogenetic tree; \( r_b \): cumulative genetic distance between the sequences; Snn: Nearest-neighbor statistic; F_St: Wright’s measure of population subdivision.
None of the statistical tests reported here were adjusted for multiple comparisons. While it is customary to adjust P-values for multiple comparisons when making positive claims, our use of uncorrected P-values is conservative with respect to the negative conclusions in this study (i.e., that compartmentalization was minimal).

**Diversity of HIV-1 gp160 env sequences from RNA in serum, PBMC proviral DNA and clonal HIV-1 variants**

We next compared the mean pairwise genetic distance per time point (diversity) within the viral gp160 env sequences derived from serum-RNA, PBMC-DNA, and CV (Figure 6.5, and summary in Table 6.2 and Supplementary Table S6.2).

Sequence diversity within serum-RNA, CV, and PBMC-DNA, which was compared at time points where sequences from all three sources were available, was similar at 1 of 3 time points in patients A and D and at 2 of 4 time points in patients B and C (Table 6.2). In patient A and C, sequence diversity was significantly lower in PBMC-DNA at one of the time points (first and fourth time point, respectively). Sequence diversity in serum-RNA was significantly lower at the last time point of patient A and significantly higher at the second time point of patient C. For patient B and D, sequence diversity in serum-RNA was significantly lower at one of the time points (third and first time point, respectively) and PBMC-DNA had the highest, and CV the lowest sequence diversity at the other time point (first and third time point for patients B and D, respectively; Table 6.2).

Diversity between serum-RNA and CV could be compared at 5, 4, 4, and 3 time points for patients A, B, C, and D, respectively (Table 6.2). At 9 of these 16 time points, corresponding to 33-75% of the time points analyzed per patient (Supplementary Table S6.2), sequence diversity between serum-RNA and CV was similar.

Diversity between CV and PBMC-DNA could be compared at 4 time points for patients A, B and C and at 5 time points for patients D (Table 6.2). At 11 of those 17 time points, corresponding to 50-75% of the time points analyzed per patient, PBMC-DNA and CV had similar sequence diversity (Supplementary Table S6.2).

Diversity in serum-RNA and PBMC-DNA could be compared at 3 time points for patients A and D, and at 4 time points for patients B and C (Table 6.2). Similar sequence diversity in serum-RNA and PBMC-DNA was found at 6 of the in total 14 time points at which the comparison was performed, corresponding to 33-50% of the time points that were analyzed per patient (Supplementary Table S6.2).

Differences in sequence diversity between serum-RNA, CV and PBMC-DNA were detected in the pairwise comparisons of the different sources under study at 25-67% time points analyzed per patient. Those differences could not be attributed to a higher or lower sequence heterogeneity of one of the sources in particular, neither to any patient-specific patterns.

No correlation was found between the viral load at a certain time point and the diversity of sequences from serum-RNA, PBMC-DNA and CV (data not shown).
Divergence of HIV-1 gp160 env sequences from RNA in serum, PBMC proviral DNA and clonal HIV-1 variants

Subsequently, we compared per time point the mean pairwise genetic distance between gp160 env sequences from serum-RNA and PBMC-DNA, serum-RNA and CV, and PBMC-DNA and CV (divergence) with the diversity of the gp160 env sequences from each source separately (Figure 6.5, and summary in Table 6.2 and Supplementary Table S6.2). For patients A, C and D, the divergence between sequences from serum-RNA and CV was similar to the diversity within sequences from either serum-RNA or CV in 33-60% of the time points analyzed. For patient B, divergence between serum-RNA and CV was always

Table 6.2: Comparison of mean gp160 env pairwise genetic distances within (diversity) and between (divergence) sequences from serum-RNA, PBMC-DNA and clonal HIV-1 variants per time point and patient

<table>
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<tr>
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SE: diversity in serum-RNA; CV: diversity in clonal HIV-1 variants; PB: diversity in PBMC-DNA; SEvsCV: divergence between serum-RNA and CV; CVvsPB: divergence between CV and PBMC-DNA; SEvsPB: divergence between serum-RNA and PBMC-DNA. = indicates no significant difference between mean pairwise genetic distances; < or > indicate significant differences between mean pairwise genetic distances (P-value < 0.05); bold indicates no significant differences between any of the comparisons. * Time point with only one serum sequence available.
higher than diversity in either serum-RNA and/or CV.
Divergence between CV and PBMC-DNA sequences was similar to the diversity within the sequences from either CV or PBMC-DNA in 40-100% of the time points analyzed per patient. Divergence between sequences from serum-RNA and PBMC-DNA was higher than the diversity within serum-RNA and/or PBMC-DNA in 33-75% of the time points analyzed per patient. The increased divergence between serum-RNA and both CV and PBMC-DNA for patients A and B coincided with the presence of CXCR4-using CV and sequences with an X4 genotype in PBMC-DNA but not in serum-RNA. Indeed, sequences with an X4 signature clustered separately from R5 sequences in the ML tree (Figure 6.1 and 6.2).

Figure 6.5: Pairwise genetic distances per time point within (diversity) and between (divergence) gp160 env sequences from viral RNA in serum (SE), PBMC proviral DNA (PB) and clonal HIV-1 variants (CV)
Mean pairwise distances were calculated per time point and compared using the Mann-Whitney test for independent samples. P-values < 0.05 were considered significant. Patients A (A), patient B (B), patient C (C), patient D (D).
Figure 6.6: Percentage of predicted R5/CCR5-using variants and predicted X4/CXCR4-using variants in serum RNA (SE), PBMC proviral DNA (PB) and clonal HIV-1 variants (CV). All CV were tested for the ability to use CXCR4 in the MT-2 assay and results were confirmed with co-receptor use prediction based on the V3 amino acid sequences and the PSSM matrix and using the geno2pheno[coreceptor] method (FPR = 5%). The same prediction methods were used to predict co-receptor use of viral sequences obtained from viral RNA in serum or from proviral DNA in patient PBMC. Numbers on bars indicate percentages of predicted R5/CCR5-using variants and predicted X4/CXCR4-using variants per time point.
Divergence between serum-RNA and both CV and PBMC-DNA at the first two time points of patient B could be attributed to the separate clustering of most of the early serum-RNA sequences in the ML tree (Figure 6.2).

**Co-receptor use of clonal HIV-1 variants and predicted co-receptor use of sequences derived from viral RNA in serum and PBMC proviral DNA**

CXCR4 usage of CV was determined by their ability to replicate in the MT-2 cell line and confirmed with prediction tools that are based on V3 amino acid sequences. The same prediction tools were used to predict co-receptor usage for viral sequences obtained from serum-RNA and PBMC-DNA.

Co-receptor use of CV and viral sequences derived from serum-RNA and PBMC-DNA was compared for patients who harbored both CCR5- and CXCR4-using variants (patients A and B, and four additional patients (E, F, G and H) from whom gp120 (C2-C4) envelope unpublished sequences from serum-RNA and CV were available (see Supplementary Table S6.1 and Supplementary Material and Methods)). Figure 6.6 shows percentages and absolute numbers of R5/CCR5-using and X4/CXCR4-using variants in serum-RNA and CV for all six patients and additionally in PBMC-DNA for patients A and B. Absence or underrepresentation of X4 variants in serum was observed in patients from whom only low numbers of clonal CXCR4-using HIV-1 variants could be isolated (patients A, B, E and F). Patients A, B and E completely lacked X4 en sequences in serum (Figure 6.6A, 6.6B and 6.6C). For patient F, X4 sequences were found in serum at the time point close to the estimated emergence of CXCR4-using variants but were no longer detected in later stages of infection (Figure 6.6D). Interestingly, in patients A and B, X4 sequences were detected in PBMC-DNA at the same time points at which CXCR4-using CV could be isolated. Patients from whom higher numbers of CXCR4-using clonal HIV-1 variants could be isolated (patients G and H; Figure 6.6E and 6.6F) had similar percentages of X4/CXCR4-using variants in serum and CV.

**DISCUSSION**

The cell-free and cell-associated HIV-1 pool in peripheral blood may potentially differ in genetic composition as the virus variants from plasma are generally considered to represent the recently produced virus population in vivo, while PBMC are considered to harbor a mix of recently produced viruses and archived viruses. To address this, we compared evolution and genetic variability of cell-associated virus (proviral DNA from PBMC (PBMC-DNA) and replication competent clonal HIV-1 variants (CV) isolated from PBMC) and cell-free virus in serum (serum-RNA). Phylogenetic analysis of gp160 env nucleotide sequences revealed that sequences derived from serum-RNA, PBMC-DNA, and CV from the same time point generally clustered together and that divergence to the most recent common ancestor was in general greater
for sequences collected at later time points of infection, independently of the source from which they were obtained. Intermingling of sequences from the three sources was observed in all patients, although segregation of sequences from serum-RNA or PBMC-DNA was found at some time points. Firstly, in patients B and C, some of the serum-RNA sequences evolved independently with no descendents at late stages of the infection, suggestive for negative selection of these viruses or only transient production of these viruses from another body compartment. Secondly, in patients A and B who showed emergence of CXCR4-using variants, the X4/CXCR4-using variants, which clustered separately from the R5/CCR5-using variants in the phylogenetic tree, were detected in patient PBMC-DNA and CV but not in serum-RNA. The segregation between sequences from serum and PBMC-DNA and/or CV after the emergence of CXCR4-using variants in these patients could be attributed to the absence of X4 variants in serum. Finally, a low degree of intermingling of viral sequences from PBMC-DNA with sequences of the other two sources was observed at time points where PBMC-DNA sequences were very homogeneous. This could either reflect low sequence diversity in PBMC-DNA at specific time points or limitations in the detection of minor variants.

In 3 out of 4 patients (A, C and D), we found evidence for frequent gene trafficking between serum-RNA, PBMC-DNA and CV, albeit that compartmentalization between PBMC-DNA and either serum-RNA or CV was borderline significant for patients A and C, which coincided with a certain degree of segregation between some of the sequences from PBMC-DNA and the other sources in the phylogenetic trees. Overall however, our data support that in each of these patients, sequences from the three sources belonged to the same viral population. In patient B, we detected a restriction of gene trafficking between serum-RNA and both PBMC-DNA and CV. This restriction is probably the result of negative selection of most of the early viruses in serum, in combination with the differential evolution of viral coreceptor use in serum and PBMC. This implies that the cell-free (serum) and cell-associated (PBMC) viral quasispecies can occasionally be different and therefore not always reflecting the same fraction of viruses present in peripheral blood at a certain time point.

Previous studies have shown the effect of PBMC cultures for bulk virus isolation on the composition of virus populations 15-18. While some argued that a minor HIV-1 variant present in vivo 16 or a variant not even detected in the patient 15 could dominate the co-culture in vitro, others found that the major variant present in uncultured PBMC was the one persisting in the culture 18. Indeed, bulk virus isolation in vitro seems to select for one or few HIV-1 variants that have optimal fitness for replication in PBMC in vitro. Although the clonal virus isolation procedure allows for the isolation of multiple variants, there is still the concern that only a fraction of the virus variants originally present in vivo may be selected and that novel mutations may occur during culturing of the virus. We here show that CV exhibit a sequence variability similar to contemporaneous viral populations obtained from viral RNA in serum and PBMC proviral DNA. When diversity between the three different
sources differed, this was not necessarily due to a higher or lower heterogeneity of sequences from CV. Moreover, significant divergence was found between CV and the viral sequences derived from serum-RNA and PBMC-DNA, but comparable differences in divergence were also observed between serum-RNA and PBMC-DNA. Therefore we conclude that CV are not a specific selection from the virus pool in blood at the moment of sampling and that the sequence variation that may have been introduced during culture in CV is limited. We are aware that with the population sequencing methodology not all viral variants present in the sample may have been detected and that we therefore may have underestimated the actual sequence variability in PBMC-DNA and serum-RNA. However, our approach at least demonstrates a similar composition of the CV and the dominant virus populations in serum-RNA and PBMC-DNA.

Differences in the genetic composition of virus populations from serum-RNA and PBMC-DNA have mostly been reported for patients receiving HAART. Drug-resistance mutations can be detected in plasma before their occurrence in PBMC. More importantly, the recovery of replication-competent wild-type HIV from PBMC despite prolonged suppression of plasma viremia, suggested a reservoir of archived viruses in PBMC \(^{41,42}\). In our present study, only a few sequences from PBMC-DNA and CV, and interestingly in two patients also from serum-RNA, were identified as potentially archived viruses. This suggests that in the absence of therapy, PBMC proviral DNA reflects mostly the actively replicating virus population and that CV can be considered an accurate reflection of the replication competent viruses at a given moment, not excluding that a low percentage of archived latent viruses may be recovered with the clonal virus isolation procedure.

Our observation that CXCR4-using variants may be more common in PBMC than in serum-RNA is in agreement with a previous cross-sectional study from Verhofstede et al. \(^{43}\). We confirm and extend those observations by showing in longitudinal samples that X4 variants were not always detected in serum when only low numbers of CXCR4-using CV were obtained. We have previously reported that early CXCR4-using HIV-1 variants have a higher sensitivity to antibody neutralization than their co-existing CCR5-using variants \(^{44}\) and several studies have suggested that HIV-specific immune responses may promote the preferential survival of CCR5-using strains \(^{45-48}\). This may suggest that a cell-free state of CXCR4-using viruses is incompatible with neutralizing humoral immunity, which could explain their absence or underrepresentation in serum and their restriction to cell-to-cell spread in PBMC. As the contribution of CXCR4-using HIV-1 to the total virus population may increase over time \(^{49}\), the equal distribution of X4 sequences in serum-RNA and CV in individuals with higher numbers of CXCR4-using variants may reflect the selection of neutralization resistant CXCR4-using variants that can persist in plasma.

The major advantage of working with replication competent CV is that biological properties of the virus can be studied in the context of the original genetic background and the complete viral genome, which obviously is not the case with cloned viral gene fragments.
from plasma in the background of a molecular HIV-1 clone. Our present study shows that clonal HIV-1 variants isolated from PBMC may equally represent the viral quasispecies in blood as sequences obtained from serum and PBMC proviral DNA. However, certain selective forces may drive differential evolution of the cell-free and cell-associated virus pool, in which case, sequences from both sources would be ideally required to obtain a more complete picture of the viral quasispecies in peripheral blood \textit{in vivo}.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Chapter 6

Supplementary material

Primers used for sequencing of gp160 env from clonal HIV-1 variants
Seq2 (rev) (5’-TCTCTCCATATCCTCCTCCAGGTC-3’),
Seq3 (fw) (5’-ATTGGGATCAAAGCCTAAAGCCATG-3’),
Seq4 (rev) (5’-CTTGTATTGTTGTTGGGTCTTGTAC-3’),
Seq5 (fw) (5’-GTCAACTCAACTGCTGGTTAAATGGC-3’),
Seq6 (rev) (5’-ATCTAATTGTGGCACTGATGGGAGG-3’),
env9 (rev) (5’-ACAGGCTGTGTAATGACTGA-3’),
env1aTOPO (fw) (5’-CAGGGCTTAGGCATCTCCTATGCGAGGAAGAA-3’),
PSC (fw) (5’-CCTCAGGAGGGACCCAG-3’),
PSH (rev) (5’-CCATAGTGCTTCCTGCTGCT-3’),
Han15 (fw) (5’-ACCAAGGAAAGAAGAGAGTGG-3’),
env5 (rev) (5’-GTTGAATATCCTGCCCTAATTCTA-3’),
envM (fw) (5’-CCACAAAATGACCCCATTTATCTA-3’)
Han16 (rev) (5’-TTCATTCTTCTCCCTACAGTACGG-3’).

Primers used for sequencing of gp160 env PCR products from serum and PBMC
M13F (fw) (5’-GTAAAACGACGGCCAG-3’), M13R (rev) (5’-CAGGAAACAGCTATGAC3’),
Senv5 (fw) (5’-GTTACCTGTGTGGAAAG-3’), F112 (fw) (5’-CAGTACATGGAACAGGATG-3’)
and EnvSeqF (fw) (5’-TTCAGACCTGGAGGAGGARATATGA-3’).

HIV-1 RNA isolation from serum, cDNA synthesis, cloning and sequencing of gp120 (C2-C4) env PCR products for Patients E, F, G and H
Viral RNA was isolated from 140µl serum using the QIAGen Viral RNA Mini Kit. Isolated
RNA was eluted in a final volume of 50µl. Viral RNA (10µl) was reverse transcribed into
cDNA with Superscript II RnaseH Reverse Transcriptase (Invitrogen).

From the synthesized cDNA of patients E, F, G and H the gp120 env region (C2 to C4: corre-
sponding to HxB2 envelope nucleotide positions 811 to 1290) was amplified in a outer PCR
(10 independent PCR’s per sample) with primers seq2 (5’-TCTCTCCATATCCTCCTCCTCCAGGTC-3’) and seq3 (5’-ATTGGGATCAAAGCCTAAAGCCATG-3’) and a nested PCR
with primers seq5 (5’-GTCAACTCAACTGCTGGTTAAATGGC-3’) and seq6 (5’-ATCTAATTGTGGCACTGATGGGAGG-3’) (outer and nested PCR temperature program: 97°C,
Multiple bulk PCR products resulting from serum RNA were cloned in the pGEM-Teasy Vector system (Promega) and transformed into DH5α competent cells (invitrogen). A
maximum of 2 clones obtained from one independent PCR were picked, gp120 env C2-C4
region was amplified with pGEM-Teasy Vector specific primers and PCR products were
purified using EXOSAP-IT (USB) and sequenced using the nested PCR primers and the
ABI prism Big Dye Terminator v1.1 Cyclesequencing Kit (Applied Biosystems) according
to the manufacturer’s protocol. Sequencing conditions were 5’ at 94˚C, 30 cycles of 15” at 94˚C, 10” at 50˚C, 2’ at 60˚C and a 10’ extension at 60˚C. Sequences were analyzed on the Applied Biosystems/Hitachi 3130 xl Genetic Analyzer.

**DNA isolation, PCR amplification and sequencing of gp120 (C2-C4) env from clonal HIV-1 variants isolated from Patients E, F, G and H**

Total DNA was isolated from 1x10⁶ healthy donor PBMC in vitro infected with clonal HIV-1 isolates, using a modified L6 isolation method ¹. For each clonal HIV-1 variant, the gp120 env region (C2 to C4: corresponding to HxB2 envelope nucleotide positions 811 to 1290) was amplified and sequenced as described for the serum samples of those patients with the omission of the cloning steps.

**Compartmentalization tests**

Five methods (Slatkin-Maddison ², Simmonds Association Index ³, Correlation Coefficients ⁴, Wright’s measure of population subdivision (F_{ST}) ⁵,⁷ and Nearest-neighbor statistic ⁸) were used to determine compartmentalization between sequences from serum, PBMC proviral DNA and clonal HIV-1 variants isolated from PBMCs. Methods and parameters implemented in Hyphy for each method are described below:

1) Slatkin-Maddison (SM) determines the minimum number of migration events between the separated populations based on the tree topology. Statistical support is based on the number of migration events that would be expected in a randomly structured population, derived by permuting sequences between compartments. 1000 permutations were used in our analysis.

2) Simmonds Association Index (AI) assesses the degree of population structure by weighting the contribution of each internal node based on its depth in the tree. 10 relabelings per sample and 1000 tree bootstrap samples were used in our analysis.

3) Correlation coefficients (r and r_b) correlate distances between two sequences in a phylogenetic tree with the information about whether or not they were isolated from the same compartment. The distance between sequences can be either the number of tree branches separating the sequences (r_b) or the cumulative genetic distance between sequences (r). To assess whether the computed coefficient was statistically significant, we estimated the distribution of these coefficients by permuting 1000 times sequences between compartments and 10 relabelings per samples were used.

4) Wright’s measure of population subdivision (F_{ST}) compares the mean pairwise genetic distance between two sequences sampled from different compartments to the mean distance between sequences samples from the same compartment. Statistical significance is derived via a population-structure randomization test. This score is calculated using two estimates of F_{ST} ⁶,⁷ and an estimate of K_{ST} ⁸. Distance matrices were calculated under the HKY85 substitution model.
5) Nearest Neighbor Statistic (Snn) measures how often the nearest neighbors of each sequence were isolated from the same or different compartments. Distance matrices were calculated under the HKY85 substitution model.

REFERENCES
Supplementary Figure S6.1
### Suplementary Table S6.1: Patients, time points and number of sequences analyzed

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<sup>a</sup> time to emergence of CXCR4-using viruses (estimated date between last negative and first positive MT-2 test).

<sup>b</sup> sequences obtained from viral RNA from serum.

<sup>c</sup> sequences obtained from clonal HIV-1 variants isolated from patient PBMC.

<sup>d</sup> sequences obtained from patient PBMC proviral DNA.

R5: number of sequences with predicted and/or experimentally tested CCR5 use.

X4: number of sequences with predicted and/or experimentally tested CXCR4 use.
Supplementary table S6.2: Comparison of mean gp160 env pairwise genetic distances within (diversity) and between (divergence) sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants per patient

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SE: diversity in serum-RNA; CV: diversity in clonal HIV-1 variants; PB: diversity in PBMC-DNA; SEvsCV: divergence between serum-RNA and CV; CVvsPB: divergence between CV and PBMC-DNA; SEvsPB: divergence between serum-RNA and PBMC-DNA. = indicates no significant difference between mean pairwise genetic distances; < or > indicate significant differences between mean pairwise genetic distances (P-value < 0.05).

<sup>a</sup> tp3 was not included because only 1 serum-RNA sequence was available.

<sup>b</sup> tp5 was not included because only 1 serum-RNA sequence was available.
Rapid escape from preserved cross-reactive neutralizing humoral immunity without loss of viral fitness in HIV-1 infected progressors and long-term non-progressors
ABSTRACT
A substantial proportion of HIV-1-infected individuals has cross-reactive neutralizing activity in serum, with a similar prevalence in progressors and long-term non-progressors (LTNP).
Here, we studied whether disease progression in the face of cross-reactive neutralizing serum activity is due to fading neutralizing humoral immunity over time or to viral escape.
In three LTNP and three progressors, high-titer cross-reactive HIV-1-specific neutralizing activity in serum against a multiclade pseudovirus panel was preserved during the entire clinical course of infection, even after AIDS diagnosis in progressors. However, while early HIV-1 variants from all six individuals could be neutralized by autologous serum, the autologous neutralizing activity declined during chronic infection. This could be attributed to viral escape and the apparent inability of the host to elicit neutralizing antibodies to the newly emerging viral escape variants. Escape from autologous neutralizing activity was not associated with a reduction in the viral replication rate in vitro. Escape from autologous serum with cross-reactive neutralizing activity coincided with an increase in the length of the variable loops and in the number of potential N-linked glycosylation sites in the viral envelope. Positive selection pressure was observed in the variable regions in envelope, suggesting that, at least in these individuals, these regions are targeted by humoral immunity with cross-reactive potential.
Our results may imply that the ability of HIV-1 to rapidly escape cross-reactive autologous NAb responses without the loss of viral fitness is the underlying explanation for the absent effect of potent cross-reactive neutralizing humoral immunity on the clinical course of infection.
INTRODUCTION

The need for an effective vaccine to prevent the global spread of human immunodeficiency virus type 1 (HIV-1) is well recognized. The ability to elicit broadly neutralizing antibodies (BrNAbs) is believed to be key to a successful vaccine, ideally to acquire protective immunity, or, alternatively, to achieve a non-progressive infection with viral loads sufficiently low to limit HIV-1 transmission 1,2.

During natural infection, antibodies that are able to neutralize autologous virus variants are elicited in the majority of HIV-1 infected individuals. Early in infection, these neutralizing antibodies (NAbs) are mainly type-specific, due to the fact that they are primarily directed against the variable domains in the viral envelope, and allow for the rapid escape of HIV-1 from antibody neutralization 39. Escape from type-specific neutralizing humoral immunity has been associated with enormous sequence variation, particularly in variable loops 1 and 2 (V1V2) of the envelope protein where large insertions and deletions are observed, as well as with changes in the number of potential N-linked glycosylation sites (PNGS) in the envelope protein 3,5,9,16. The rapid escape of HIV-1 from autologous type-specific Nabs seems to be the underlying explanation for the absent correlation between autologous humoral immunity and HIV-1 disease course. Furthermore, we recently observed that the changes in envelope that are associated with escape from autologous neutralizing humoral immunity do not coincide with a loss of viral fitness 17, providing an additional explanation for the lack of protection from disease progression by the autologous type-specific NAb response.

In the last couple of years, the focus of research has shifted towards neutralizing humoral immunity with cross-reactive activity, defined as the ability to neutralize a range of heterologous HIV-1 variants from different subtypes. It has become apparent that about one-third of HIV-1 infected individuals develop cross-reactive neutralizing activity in serum. However, the prevalence of cross-reactive neutralizing activity in serum was similar for HIV-infected individuals with a progressive disease course and long-term non-progressors (LTNP) 18-21.

Here, we studied the underlying explanation for this observation in three LTNP and three progressors who all had high-titer cross-reactive neutralizing activity in serum within two to four years post-seroconversion (SC). In all individuals, we observed that the potent and cross-reactive neutralizing immunity was preserved during the entire course of infection. However, the presence of cross-reactive neutralizing activity in serum did not prevent rapid viral escape from humoral immunity, which coincided with changes in envelope similar to those described for escape from type-specific autologous humoral immunity. Although broadly neutralizing antibodies are assumed to target the more conserved epitopes that may lie in crucial parts of the viral envelope, escape from cross-reactive neutralizing activity did not coincide with a loss in viral fitness. Our findings underscore that vaccine-elicited cross-reactive neutralizing immunity should protect against HIV-1 acquisition, as protection
from disease progression, even by humoral immunity with strong cross-reactivity, may be an unachievable goal.

**Materials and methods**

*Participants and viruses*

The six individuals studied here were selected from the Amsterdam Cohort Studies (ACS) on HIV and AIDS in homosexual men. Long-term non-progressors (LTNP) were defined as HIV-1-infected individuals who have ≥10 years of asymptomatic follow-up with stable CD4 counts that are still above 400 cells/µl in the 9th year of follow-up. Typical progressors were defined as HIV-1-infected individuals who progressed to AIDS within 7 years after SC. All individuals were infected with HIV-1 subtype B. Five individuals were seropositive at entry into the cohort studies (seroprevalent cases with an imputed SC date on average 18 months before entry into the cohort 22,23), while participant H18969 seroconverted during active follow-up 9. None of the individuals received combination anti-retroviral therapy during the follow-up period for this study.

Clonal virus variants were obtained as previously described 24,25. For further study, we selected a maximum of 5 virus variants per individual per time point to be tested for autologous neutralization sensitivity. Viruses were selected on the basis of their replication capacities, to get a mix of different virus variants that had coexisted in vivo. To prevent a change in neutralization sensitivity of the virus variants during in vitro culture, the number of virus passages in peripheral blood mononuclear cells (PBMC) was kept to a minimum 26.

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.

**U87/pseudovirus assay for testing of HIV-1 cross-reactive neutralizing activity in serum**

Sera from these six individuals were tested for neutralizing activity in a pseudovirus assay developed by Monogram Biosciences. The tier 2-3 virus panel that we used for determining cross-neutralizing activity in serum consisted of HIV-1 pseudoviruses from subtypes A (n=5), B (n=6), C (n=7), and D (n=5). Viruses were obtained recently after transmission or during the chronic phase of infection, and included both moderately neutralization sensitive and neutralization resistant primary HIV-1 variants, based on previously determined neutralization sensitivities to subtype B sera and monoclonal antibodies b12, 2G12, and 4E10 21,27,28. Not all sera were tested against all viruses of the panel. Pseudotyped viral particles were produced by cotransfecting HEK293 cells with an expression vector carrying the HIV-1 derived gp160 gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucPCNDO-AU3). Forty-eight hours after transfection, pseudovirus stocks were harvested and small aliquots were tested for infectivity using U87 target cells expressing
CD4, CCR5, and CXCR4. Pseudovirus stocks were tested and normalized for infectivity prior to testing in the neutralization assay.

A recombinant virus assay involving a single round of virus infection was used to measure cross-neutralization activity of the sera\textsuperscript{[29]}. Diluted pseudoviruses were incubated for 1 hour at 37 °C with serial dilutions of serum after which the U87 target cells were added. The ability of participant 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 amphotropic murine leukemia virus envelope proteins gp70SU and p15TM (aMLV). Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC\textsubscript{50}). Neutralization titers were considered positive if they were 3 times greater than the negative aMLV control and were ≥100. The lowest serum dilution used in the assay was 1:40.

**PBMC-based assay for testing HIV-1 autologous neutralizing activity in serum**

Clonal virus variants of participants were tested for their relative neutralization sensitivities against autologous serum and pooled sera from healthy, uninfected individuals. 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 5x10\textsuperscript{6}/ml. After inoculation, the cells (1x10\textsuperscript{6}/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 was incubated for 1 hour at 37 °C with decreasing concentrations of the serum (starting concentration 1:50) in 96-well microtiter plates. Subsequently, 1x10\textsuperscript{6} PHA-stimulated PBMC were added to the mixtures of virus with serum. After 4 hours of incubation, PBMC were washed once in 100 μl phosphate-buffered saline after which fresh medium was added. On day 11, virus production in culture supernatants was analyzed in an in-house p24 antigen capture enzyme-linked immunosorbent assay\textsuperscript{[30]}. Background measurements were performed using pooled sera from uninfected individuals and neutralization titers were expressed as the reciprocal serum dilution that established 50% inhibition (IC\textsubscript{50}) of virus infection. Experiments were performed in triplicate. When possible, 50% inhibitory concentrations (IC\textsubscript{50}) were determined by linear regression. For calculation of IC\textsubscript{50} values for viruses that were not inhibited by the 1:50 serum dilution, we assumed that 50% inhibition would have occurred at a 1:25 serum dilution.
Preparation of chimeric viruses

To exclude an effect of additional mutations in other genes than Env on the viral replication rate, we generated a panel of chimeric NL4-3 viruses, in which the original envelope was replaced with the envelopes of virus variants that were isolated from our participants. For each time point, envelopes from a minimum of 2 and a maximum of 8 viruses were analyzed. Env fragments from HXB2 nucleotides (nt) 5658 to 9171 were amplified by PCR using Expand High Fidelity PCR System (Roche Applied Science). Chimeric NL4-3/Env viruses were produced by homologous recombination of the Env PCR products with a pNL4-3 vector (a kind gift from 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.

Sequence analysis

The HIV envelope gp160 gene was PCR amplified from DNA isolated from PBMC that were infected in vitro with a single clonal HIV-1 variant and subsequently sequenced as described previously. The nucleotide sequences of all virus clones from an individual 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. Genetic analyses were performed on gp160 sequences starting at nucleotide position 91, which excludes the Env signal peptide. PNGS were identified using N-glycosite at the HIV database website (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). Net charges of gp160 were calculated by counting all charged amino acid residues per sequence, where residues R and K counted as +1, H as +0.293, and D and E as -1. Nonsynonymous substitution (dN) and synonymous substitution (dS) rates for the different regions of env were calculated using Synonymous Nonsynonymous Analysis Program (https://hiv.lanl.gov/content/sequence/SNAP/SNAP.html). dN/dS ratios were calculated between successive time points by averaging the dN/dS ratios between all individual pairs of env sequences from the two time points. Positively selected codons were identified using DataMonkey (http://www.datamonkey.org) with the REL, FEL and SLAC method and were assumed to be truly positively selected if two methods were significant (P value < 0.05). To ensure a correct calculation of dN/dS ratios and positive selection in the variable loops, the codon alignments of these regions were corrected manually. Codons containing indels were excluded in this method.
The sequences used in this study have been deposited in GenBank under accession numbers EU744055 to EU744096 and GU455425 to GU455525.

Characterization of HIV-1 replication kinetics

Replication kinetics of the clonal virus variants were determined using chimeric NL4-3/Env viruses on pooled PBMC which were obtained and stimulated as described above. 2x10⁶ PHA-stimulated PBMC were inoculated with 500 50% tissue culture infective doses of a given chimeric NL4-3/Env HIV-1 variant in a volume of 2 ml at 37 °C for 2 hours in a shaking water bath. Subsequently, cells were washed with 10 ml Iscove’s modified Dulbecco medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and resuspended at a concentration of 1x10⁶ cells/ml for culture. Fresh PHA-stimulated PMBC (1x10⁶) in a volume of 1 ml were added at days 5 and 8. Cultures were maintained for 11 days. 75 µl of supernatant for determination of p24 antigen production were harvested each day. The concentration of p24 in all samples was determined at the same time using an in-house p24 antigen capture enzyme-linked immunosorbent assay. P24 production per ml supernatant was determined and corrected for the differences in volume of culture supernatant. Per individual, the period of logarithmic expansion of viral p24 production was determined and only this timeline was used for further analyses.

Statistical analysis

Statistical analyses were performed using the SPSS 16 software package. Changes in replication kinetics were compared using an unpaired two-sample t test. Changes in the length and the number of PNGS in Env were assessed using the Kruskall-Wallis analysis of variance.

RESULTS

Longitudinally preserved cross-reactive neutralizing serum activity in three LTNP and three progressors

We previously demonstrated a similar prevalence of cross-reactive neutralizing activity in sera of LTNP and progressors at time points relatively early in infection. Here, we first wished to study whether a progressive disease course was associated with a more rapid loss of cross-reactive neutralizing serum activity at the later stages of disease. To this end, we selected three LTNP and three progressors from the Amsterdam Cohort Studies on HIV infection and AIDS, for whom we previously established cross-reactive neutralizing activity in serum samples that were obtained at around years two and four post-SC. For these patients, we analyzed cross-reactive neutralizing activity in sera that were obtained at multiple time points during the course of infection, up to the moment of clinical AIDS diagnosis or initiation of HAART in the three progressors and in one LTNP who ultimately progressed to AIDS, or until end of follow-up in the other two LTNP (Figure 7.1). HIV-1
specific neutralizing activity was measured in a cell-based infectivity assay using a panel of 23 recombinant viruses pseudotyped with envelope proteins from HIV-1 subtype A, B, C, and D. Due to the limited availability of serum, some sera were only tested against a subset of this virus panel. HIV-specific cross-reactive neutralizing activity, defined as an IC$_{50} \geq 100$ against at least 50% of the viruses from 3 or more subtypes, was observed in sera from all six individuals. For participant H18969, cross-reactive neutralizing serum activity developed as early as 12 months post-SC (Figure 7.2). In contrast, serum from participant H19663 did not show cross-reactive neutralizing activity until 59 months post-SC, although serum obtained 29 months post-SC from this participant was already able to neutralize virus variants from different subtypes (Figure 7.2). In the remaining 4 participants, cross-reactive neutralizing serum activity was observed after approximately 30 months post-SC (Figure 7.2). However, serum samples from earlier time points were not available for these 4 participants, indicating that cross-reactive neutralizing activity could have been present earlier in infection.

Figure 7.1: CD4$^+$ T-cell count, viral RNA load and antiretroviral treatment during the course of infection of three LTNP (top) and three progressors (bottom)

The CD4$^+$ T-cell count are shown in black with the legend on the left y-axis, while the viral RNA load are indicated in gray with the legend on the right y-axis. The detection limit for the measurement of RNA load was 1000 copies/ml plasma, which decreased to 400 copies/ml plasma later in time (for participant H19956 from 200 months onwards). The length and type of antiretroviral therapy are indicated at the top of each diagram.
exception, cross-reactive neutralizing serum activity was conserved longitudinally in both LTNP and progressors. Neutralizing serum titers increased over the course of infection until the end of follow-up in 2 LTNP, or until around the moment of clinical AIDS diagnosis for the 4 participants who developed AIDS. After clinical AIDS diagnosis, cross-reactive neutralizing serum activity declined, although the breadth of neutralization was preserved (Figure 7.1 and 7.2).

Decreasing neutralizing humoral immunity against autologous HIV-1 during the course of infection

The observation that cross-reactive neutralizing serum activity was preserved during the course of infection in both LTNP and progressors excludes the possibility that loss of humoral immunity precedes disease progression. To investigate whether viruses from LTNP

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**Figure 7.2: Breadth and potency of HIV-1 specific neutralizing activity in sera obtained during the course of infection from three LTNP (top) and three progressors (bottom)**

IC₅₀ values, given as the reciprocal serum dilution determined using a U87-based neutralizing assay, are shown for serum samples obtained during the course of infection against a panel of 23 heterologous virus variants. Due to limiting amounts of serum, some sera were only tested against a subset of this virus panel. In the left columns, a description of the virus panel is given; the tier 2-3 virus panel consisted of HIV-1 pseudoviruses from subtype A, B, C and D. The references panel (bottom part) included strains 1196, BaL, JRCSF, NL4-3, and SF162. As a negative control (NC), the amphotropic murine leukemia virus was used. IC₅₀ titers ≥ 1:100 and exceeding three times the background reading for that sample are indicated in gray. n.d., not done.
and progressors showed a difference in their ability to escape from autologous humoral immunity, we next analyzed the efficacy of neutralizing serum activity against autologous virus variants.

Clonal HIV-1 variants were isolated from PMBC that were obtained at approximately the same time points at which the sera were collected. Although 1-5 clones per time point were isolated from earlier time points in participant H19956, attempts to isolate clonal HIV-1 variants from PBMC that were obtained at time points after 150 months post-SC were not successful. From participant H19642, both R5 and X4 HIV-1 variants were isolated at the time point just before clinical AIDS diagnosis, while from earlier time points only R5 variants were obtained. From participant H19554, both R5 and X4 HIV-1 variants were isolated at the time points around 5.5 and 7 years after SC, but only R5 HIV-1 variants were isolated at the time point after clinical AIDS diagnosis.

For each individual, autologous neutralizing activity in sera obtained at or close to the time points of virus isolation were measured against a maximum of five randomly selected clonal HIV-1 variants per time point, both R5 and X4 HIV-1 variants when applicable. The number of HIV-1 variants that could be tested was limited by the amount of participant serum that was available. Neutralization of autologous virus variants was observed in all six individuals, although the level of neutralization was diverse (Figure 7.3). In agreement with findings by

![Figure 7.3: Development of autologous humoral immune responses during the course of infection in three LTNP (top) and three progressors (bottom)](image)

Average IC50 values, determined by linear regression, of ≤5 virus variants per time point are indicated. The time points of virus isolation are indicated in the top right corner of each panel. Bars with identical shading represent inhibition of virus isolates from one time point by sera of different time points (as indicated on the x-axis). The dashed lines represent background measurements using pooled sera from healthy uninfected individuals. Note that the maximum value on the y axis in the graph of participant H19642 and H18969 are higher than in the other graphs. IC50, 50% inhibitory concentration; mo, months; SC, seroconversion.
others, virus variants were poorly neutralized by contemporaneous serum and sera from earlier time points, suggestive of viral escape. In general, the neutralizing titer in serum was highest against the earliest virus variants and was much less potent against virus variants from subsequent time points. Moreover, this limited autologous neutralizing activity against early viruses was lost after AIDS diagnosis in those individuals who ultimately progressed to AIDS. For participant H19956, we observed a different pattern of neutralization, although it should be mentioned that the viruses from this participant were isolated from much earlier time points than the sera that were available for testing. For this reason, titers against all viruses were somewhat higher than what was observed in the other patients, and the highest titer was observed for the last serum sample tested against an earlier virus variant. However, only a single virus variant was obtained from the 123 and 146 months post-SC time points, respectively, not allowing firm conclusions on the effect of humoral immunity in this individual.

Overall, for viruses from the same time point, neutralizing titers in serum varied only minimally. In all six individuals, autologous neutralizing activity was lost already in the asymptomatic phase of infection, before clinical AIDS was diagnosed. Moreover, we did not observe any difference between LTNP and progressors in autologous neutralizing activity. Escape from autologous neutralization did not coincide with changes in plasma viral RNA load and/or CD4+ T-cell counts (Figure 7.1 and 7.3). We also did not observe a difference in neutralization sensitivity between R5 and X4 HIV-1 variants.

Our data show that autologous neutralizing antibody responses could no longer be mounted later in infection, and that the autologous neutralizing activity that was elicited early in infection diminished over time, while at the same time heterologous responses were preserved.

**Evolution of the envelope protein during the course of infection in individuals with cross-reactive neutralizing activity in serum**

Next, we analyzed the molecular changes in the viral envelope during the clinical course of HIV-1 infection that coincided with escape from neutralizing humoral immunity with cross-reactive neutralizing activity. To this end, full-length gp160 sequences were generated from a median of five virus variants (range 1-10) per time point (Table 7.1). Phylogenetic analysis of all sequences using the neighbor-joining method revealed clustering of sequences per individual and excluded superinfection and contamination of samples (data not shown). Escape of HIV-1 from type-specific NAbs has been associated with increases in the length of the viral envelope and the number of potential N-linked glycosylation sites in Env. For the virus variants that were isolated from the six individuals in our present study, we observed an increase in length of gp160 during the course of infection. For viruses of participants H19642 and H18969, this extension of the length of the envelope protein reached a plateau, while the envelope length of viruses from participants H19289 and
Table 7.1: Envelope characteristics of the isolated virus variants per individual per time point

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<th># virus</th>
<th>Length gp160</th>
<th>PNS gp160</th>
<th>PNS C region</th>
<th>PNS V region</th>
<th>dN/dS C region gp120</th>
<th>dN/dS V region gp120</th>
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</table>

The average sequence characteristic for all viruses from one time point is presented. Standard deviations are given between brackets. Changes in sequence characteristic over the course of infection within each individual were calculated using the Kruskall-Wallis test. The dN/dS ratios are a comparison between viruses of that time point and viruses of the previous time point.
H19544 decreased at later time points (Table 7.1). The plateau or decrease in the length of the envelope protein coincided with fading autologous neutralizing activity in these participants (Figure 7.3). The changes in gp160 length could be completely attributed to the variable regions, except for viruses from participant H19663, in which minor insertions in C3 were observed. Insertions and deletions were observed in V1 and V4 for viruses from all participants, while additional changes in the other variable regions of gp120 were observed for viruses from some participants (data not shown).

A similar pattern of change over the course of infection was observed for the number of PNGS. The changes in PNGS in gp160 of all individuals over time were caused by the acquisition and/or loss of PNGS in both the constant and variable regions of gp160 (Table 7.1). For all individuals, the number and/or location of the PNGS in the C3 and V1V2 region of gp120 changed over time. Moreover, additional changes in other regions of the envelope protein were observed in viruses from participants H19642, H19956, H19663, and H19554 (data not shown). Changes in gp160 length and PNGS did not always occur simultaneously in time. For example, in viruses from participant H19642, the number of PNGS decreased already from 4 years post-SC onwards, while the average length of gp160 still increased (Table 7.1).

Changes in the net charge of the V1V2 loop during infection have previously been reported to be correlated with higher neutralizing titers. Apart from an increase in the net charge of V2 over time in viruses from all individuals, we did not observe any uniform changes in the envelope net charge over the course of infection (data not shown).

To characterize regions in the envelope protein that were positively selected over the course of infection, we calculated the selection pressure per codon using virus variants from all different time points for each individual, as well as the dN/dS ratio for the variable and constant regions between virus variants from successive time points. Positively selected codons were observed in all regions of gp160, and did not reveal specific mutations that correlated with neutralization sensitivity. However, dN/dS ratios were highest for the variable regions, suggesting that the selection pressure was strongest in these regions. Moreover, evidence for positive selection of the constant regions was absent in viruses from all participants except for viruses obtained from participant H18969 between 0 and 4 years post-SC (Table 7.1). dN/dS ratios decreased over time and were similar for viruses from LTNP and progressors.

Overall, we did not observe any differences in length, number of PNGS, or net charge between gp160 of viruses from LTNP and progressors. In addition, similar regions of the viral envelope showed evidence of positive selection. These results indicate that the evolution of HIV-1 over the course of infection is similar in both LTNP and progressors with cross-reactive neutralizing serum activity.
Figure 7.4: Replication kinetics of clonal virus variants obtained during the course of infection from three LTNP (top) and three progressors (bottom)

Replication rates of individual chimeric NL4-3/Env variants are expressed as the p24 production during the logarithmic expansion after infection of PHA-stimulated PBMC. The horizontal lines represent the means. Note that the maximum value on the y axis in the graphs is different for each individual. SC, seroconversion.

Escape from cross-neutralizing activity does not coincide with a loss of viral replication capacity in vitro

Cross-reactive neutralizing activity is assumed to be directed against more conserved regions in the viral envelope. Escape mutations in these regions may therefore have an impact on the viral replication fitness. Here, we studied whether escape from autologous humoral immunity with cross-reactive neutralizing activity was associated with a reduction in viral replication fitness. Although the molecular changes that we observed here were similar for viruses from LTNP and progressors, this does not exclude that specific amino acid changes in LTNP viruses, or molecular changes in the background of these viruses, have a higher impact on viral replication rate than similar changes in the background of the HIV-1 variants from the progressors we studied here. Therefore, by affecting the viral replication rate, humoral immunity could still, although indirectly, contribute to the differential clinical course in LTNP and progressors. As some individuals in our study received antiretroviral monotherapy for certain periods of time, HIV-1 variants with drug resistance mutations may have been selected. To exclude an effect of these and any other mutations outside Env on the viral replication rate, we generated a panel of chimeric NL4-3 viruses in which the original envelope gene was replaced with the envelope genes of the virus variants that were isolated from our participants during the clinical course of infection. Replication kinetics were determined by the logarithmic expansion of equal viral inocula in PHA-stimulated...
PBMC and analyzed as p24 production during the period of logarithmic expansion. From participant H19956, too few clonal virus variants were available for analysis of replication rate.

Replication kinetics varied between viruses from a single individual, and even between viruses obtained from the same time point. Over the course of infection, we generally observed either stable or increasing replication rates (Figure 7.4), suggesting that escape from cross-neutralizing activity did not coincide with a reduction of the viral replicative capacity. However, replication rates of HIV-1 variants from participant H18969, decreased during the first 47 months of infection, which coincided with the presence of autologous neutralizing activity in serum (Figure 7.4). This might imply that for HIV-1 variants from this individual, an effect of NAb escape mutations on viral replication fitness cannot be excluded. We observed that an increase/decrease in replication rate did not correlate with changes in plasma viral RNA load (Figure 7.1 and 7.4) and that there was no difference in replication kinetics between R5 and X4 HIV-1 variants of these individuals.

Discussion

HIV-1-specific cross-reactive humoral immunity is assumed to be directed against relatively conserved regions on the viral envelope. As a consequence, HIV-1 may be unable to rapidly escape from cross-reactive NAb pressure, suggesting that a broad and potent humoral immune response may influence the clinical course of infection. However, we have recently demonstrated that the prevalence of cross-reactive neutralizing activity in serum is similar among HIV-infected individuals with a progressive disease course and LTNP 19. This absent correlation between disease course and cross-reactive neutralizing activity in serum could either point to fading humoral immunity in the progressive course of infection, or to viral escape from antibody pressure, as has been shown to occur in response to type-specific neutralizing humoral immunity 3-9,15,16.

In the longitudinal analysis performed in our present study, cross-reactive neutralizing humoral immunity was preserved in both LTNP and progressors, even after the moment of AIDS diagnosis in those individuals who ultimately progressed to AIDS. In contrast, autologous neutralizing activity was only observed against viruses that were isolated early in infection. Moreover, this limited autologous neutralizing activity against early viruses was lost after AIDS diagnosis. These findings not only point towards a rapid selection of HIV-1 variants that resisted the neutralizing activity in serum, it also shows the inability of the infected host to generate novel neutralizing antibody specificities against these escape variants.

One could argue that the apparent discrepancy between preserved cross-reactive neutralizing activity but fading autologous neutralizing activity could relate to differences in sensitivities of the assays used for their detection 38. Cross-reactive neutralizing activity was tested against a panel of pseudoviruses in a U87-based assay while autologous neutralizing activity was
tested in a PBMC-based assay with replicating viruses. However, we have previously shown that the relative potency of neutralizing serum activity as detected by these two assays is comparable. The different profiles of autologous versus heterologous neutralizing activity over the course of infection as observed in this study are thus likely to reflect true differences in the development and persistence of these components of neutralizing serum activity.

We recently demonstrated that escape from type-specific autologous neutralizing activity in serum did not influence the in vitro replication fitness of HIV-1. However, our observation that rapid escape of HIV-1 from autologous humoral immunity with cross-reactive neutralizing activity also had no impact on the viral replicative fitness was somewhat unexpected as BrNAbs are considered to target conserved epitopes which by definition carry crucial functions for the virus. It is tempting to speculate that replication fitness is restored by compensatory mutations that may rapidly be selected. This is currently under investigation.

Overall, the similar potency of humoral immunity, the similar dynamics of viral escape, and the absent impact of escape on the replication kinetics of viruses from both LTNP and progressors argue against a role for NAb in the clinical course of infection. In agreement, we and others have shown in comprehensive cohort analyses that the presence of cross-reactive neutralizing activity was not associated with prolonged AIDS free survival. Indeed, HIV-1 cellular immunity and host genetic background seem to have a more pronounced effect on disease progression.

Escape from autologous neutralizing humoral immunity with cross-reactive activity coincided with an increase in the length and number of PNGS of gp160 and an increase in the net charge of the V2 region. Similar changes were observed in HIV-1 variants that escaped from autologous neutralizing humoral immunity with only type-specific activity. This may either suggest that the same mechanisms apply for escape from different antibody specificities or that the relevant changes for escape from cross-reactive neutralizing antibodies are masked by changes that are selected by type-specific antibodies. Positive selection pressure was mainly observed in the variable regions of the envelope protein.

Interestingly, two novel highly potent cross-reactive neutralizing antibodies directed against a conformational epitope in the V2V3 region have recently been described, suggesting that the variable regions can indeed be targeted by cross-reactive neutralizing antibodies. We are currently studying the exact nature of the humoral immune response in the individuals in our study which will reveal whether the cross-reactive neutralizing activity is determined by a single high affinity antibody, or by a combination of multiple coexisting neutralizing antibodies directed at multiple distinct regions of the envelope. Results from these analyses will help to define which changes in the viral envelope are relevant for escape from cross-reactive neutralizing activity.

Taken together, our current findings seem to underscore the absent role for cross-reactive neutralizing humoral immunity in the protection from disease progression due to the ability
of HIV-1 to rapidly escape from this immune pressure without a loss of viral fitness. Whereas vaccine-elicited cellular immunity may be able to control viremia and thereby contribute to protection from disease progression, our results support the notion that vaccine elicited BrNAbs may only be relevant for the protection from acquisition of infection.

Acknowledgments
We thank the participants of the Amsterdam Cohort Studies for their continuous participation in the study. We are grateful to Diana Edo Matas for her help with the sequence analysis. We thank José Alcami for his kind gift of the pNL4-3 vector.

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Escape from HIV-1 cross-reactive humoral immunity
Changing sensitivity to broadly neutralizing antibodies b12, 2G12, 2F5, and 4E10 of primary subtype B HIV-1 variants in the natural course of infection
Abstract

The conserved nature of the epitopes of the four broadly neutralizing antibodies (BNAbs), b12, 2G12, 2F5, and 4E10, may imply that the sensitivity of HIV-1 for these BNAbs remains fairly constant over the course of infection. Here, we demonstrate that viruses isolated early during the course of infection were mostly sensitive to HIVIg and antibody neutralization, although variation was observed in neutralization sensitivity of coexisting viruses to the different antibodies as well as between viruses from different patients. HIV-1 resistance to HIVIg developed relatively early during follow-up in three out of five patients, while early, b12 sensitive viruses in three out of five patients were replaced by b12 resistant variants relatively late in infection. In contrast, viruses generally remained sensitive to 2F5 and 4E10 neutralization over the course of infection, although 2F5 and/or 4E10 resistant variants did emerge later in infection in four out of five patients. In most patients, HIV-1 resistance to 2F5 or 4E10 did not correlate with mutations at critical amino acid positions in their defined epitopes. Viruses resistant to 2G12-mediated neutralization were present throughout the course of infection. As viral resistance against BNAb-mediated neutralization generally developed when autologous serum neutralizing activity had faded, it seems unlikely that these changes are driven by escape from autologous humoral immunity.

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INTRODUCTION

The humoral immune response which develops in the natural course of human immunodeficiency virus type 1 (HIV-1) infection generally has limited cross-reactivity, although a subset of individuals may develop broadly neutralizing activity in serum later in infection. It is still unclear if broadly neutralizing activity in serum is the resultant of a single highly effective antibody, or of different antibody specificities which in combination give a cross-reactive neutralizing phenotype. Although a recent study points towards the latter possibility, a small number of broadly neutralizing antibodies (BNAbs) have been isolated from HIV-infected individuals, of which the four best characterized are b12, 2G12, 2F5, and 4E10.

Antibody b12 recognizes a conformational epitope which partially overlaps the CD4 binding site in gp120, whereas 2G12 is directed against a cluster of carbohydrate residues on the surface of gp120 close to the coreceptor binding domain. 2F5 and 4E10 bind to adjacent linear epitopes located within the membrane-proximal external region (MPER) of gp41. The fact that these epitopes are conserved on the viral envelope may explain the broadly neutralizing phenotype of the antibodies by which they are targeted. However, even for these BNAbs some subtype-specific neutralization patterns have been observed.

Indeed, the neutralizing potency of BNAbs b12 and 2G12 is higher against subtype B HIV-1 than to HIV-1 variants from other subtypes, which may relate to the fact that these antibodies were isolated from subtype B HIV-1 infected individuals. The MPER is more conserved between different subtypes, explaining the relatively large neutralization breadth of the anti-gp41 Abs, albeit that the potency of the 4E10 antibody is lower than that of the other BNAbs. Although the MPER is relatively conserved, the 2F5 epitope is absent in a large proportion of subtype C viruses, which explains the limited neutralizing activity of the 2F5 antibody against HIV-1 variants of this subtype.

We and others have previously analyzed primary HIV-1 from acute, early and chronic stages of infection for their sensitivities to BNAbs, and could correlate neutralization resistance to a certain BNAb with mutations in the corresponding epitope. Interestingly, neutralization resistance was also observed in the absence of mutations in the epitope, indicating that mutations outside the epitope may influence the structural context of the envelope and thereby the exposure of the epitopes for these BNAbs. Since b12, 2G12, 2F5, and 4E10 target conserved regions of the viral envelope, and considering the fact that these antibody specificities are rare, which will limit the selection pressure on these regions, one might expect that the epitopes of these BNAbs are well preserved, and that the sensitivities of virus variants throughout the clinical course of infection for these BNAbs will remain relatively constant. To study the extent of change in BNAb susceptibility of HIV-1 within an individual over the course of infection, we performed a longitudinal analysis of the sensitivities to BNAbs b12, 2G12, 2F5, and 4E10 of primary HIV-1 variants that were isolated from five typical progressors during the course of HIV-1 infection. In addition, we...
analyzed the envelope sequences for variation in the antibody epitopes that correlated with sensitivity of the viruses for the respective antibodies.

**Materials and methods**

**Patients and viruses**

The patients in our present study were homosexual male participants of the Amsterdam Cohort Studies on HIV/AIDS (ACS) who seroconverted during active follow-up and who progressed to AIDS in the presence of CCR5-using (R5) HIV-1 variants only, as shown by absent virus replication in 3-monthly performed cocultures of patient PBMC and the MT2 cell line. For all virus 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. For better readability, patient identifiers were recoded as H1 (ACH19999), H2 (ACH19542), H3 (ACH18969), H4 (ACH19768) and H5 (ACH19659), which correspond to the identifiers used in a previous study. Clonal HIV-1 variants were obtained as previously described. For further study, we selected a maximum of five virus variants per patient per time point, among which were both rapidly and slowly replicating viruses. The viral replicative capacity was defined as the first day of detectable p24 production in the micro-culture after the start of the clonal virus isolation procedure. 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.

**Neutralization assays**

Primary HIV-1 were tested for their neutralization sensitivity against HIVIg (AIDS reagent program #3957) using a TZM-bl-based assay, and against broadly neutralizing monoclonal antibodies b12 (kindly provided by D. Burton), 2G12, 2F5, and 4E10 (Polymun Scientific, Vienna, Austria) using a PBMC-based assay. Both assays were performed in triplicate, using the same clonal HIV-1 variants.

(i) **TZM-bl-based assay:** To inhibit replication of the virus variants in TZM-bl cells, a final concentration of 1 µM indinavir (AIDS reagent program #8145) was added to Iscove’s modified Dulbecco medium (Whitaker) supplemented with 10% fetal bovine serum (Hyclone), penicillin (Gibco Brl) (100 U/ml), streptomycin (Gibco Brl) (100 µg/ml), and DEAE dextran (37.5 mg/ml). From each virus isolate, a final inoculum of 20 TCID₅₀ as determined on TZM-bl cells, in a volume of 100 µl was incubated for 1 hour at 37 °C with twofold serial dilutions of HIVIg (range 15.6 – 500 µg/ml). Subsequently, the mixtures of virus with antibody were added to 104 TZM-bl cells in 100 µl medium. After 48 hours, the TZM-bl cells were washed once in 150 µl phosphate-buffered saline. Next, 25 µl freshly prepared luciferase substrate (0.83 mM ATP, 0.83 mM d-luciferin [Duchefa Biochemie B.V., Haarlem, The Netherlands], 18.7 mM MgCl₂, 0.78 µM Na₂H₂P₂O₇, 38.9 mM Tris [pH 7.8], 0.39% [vol/vol] glycerol, 0.03% [vol/vol] Triton X-100, and 2.6 µM dithiothreitol) was...
added and luminescence was measured for 1 s per well. For calculations, the background luciferase expression was subtracted from the relative light units (RLU) of the test wells. The percent neutralization was calculated by determining the reduction in luciferase expression in the presence of neutralizing agent compared to the cultures with virus only.

(ii) PBMC-based assay: Prior to the experiment, cryopreserved pooled PBMCs isolated from buffy coats obtained from 12 healthy seronegative blood donors by Ficoll-Isopaque density gradient centrifugation were thawed. The cells (5x10^6/ml) were stimulated for 3 days in IMDM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), ciproxin (5 μg/ml), and phytohemagglutinin (PHA; Welcome) (5 μg/ml). From each virus isolate, a final inoculum of 20 TCID₅₀, as determined on the same pool of PBMC, in a volume of 100 μl was incubated for 1 hour at 37 ºC with threefold serial dilutions of monoclonal antibody (range 0.034 - 25 μg/ml). Subsequently, the mixtures of virus with antibody were added to 10⁵ PHA-stimulated PBMCs in 50 μl medium, in the absence of PHA and supplemented with recombinant interleukin-2 (20 U/ml, Chiron Benelux, Amsterdam, The Netherlands) and polybrene (5 μg/ml; hexadimethrine bromide, Sigma, Zwijndrecht, The Netherlands). Virus production in culture supernatants on day 7 was analyzed by an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA) 27. 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.

Sequence analysis
Env was amplified from DNA that was isolated from healthy donor PBMC that were infected in vitro with clonal HIV-1 variants. Env PCR products were subsequently sequenced as described previously 22,28,29. Nucleotide sequences of all virus clones per individual were aligned using ClustalW in the software package of BioEdit 30, 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 were identified using N-Glycosite 31 at the HIV database website (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html).

All sequences of virus variants included in this study are available from GenBank (accession numbers EU743973 to EU744175).

Statistical analysis
For calculations and statistical analyses, viruses with IC₅₀ s of > 25 μg/ml (for monoclonal antibodies), or > 500 μg/ml (for HIVIg) were assigned a value of 25 or 500, respectively. Statistical analyses were performed in SPSS 16 software package. Longitudinal changes in neutralization sensitivity were assessed using a Kruskal-Wallis test. Differences in susceptibility between virus variants isolated from two different time points were evaluated.
RESULTS

Patients and viruses

We investigated the in vitro sensitivity for HIVIg, a pooled IgG from HIV+ individuals, and 4 broadly neutralizing antibodies (BNAbs), of subtype B HIV-1 variants that were isolated at multiple time points over the course of infection from five participants of the ACS. From these individuals, who progressed to AIDS in 7-11 years and who did not develop X4 HIV-1 variants, we have previously isolated clonal HIV-1 variants from PBMCs that were obtained at six time points covering the disease course from SC up to 2 to 3 years after clinical AIDS diagnosis. A minimum of two and a maximum of five clonal variants per time point were analyzed for their neutralization sensitivity. For patient H5, attempts to isolate clonal HIV-1 variants from PBMCs obtained at SC and at time points after AIDS diagnosis were not successful.

Figure 8.1: Sensitivity for neutralization by HIVIg, b12, 2G12, 2F5, and 4E10 of longitudinally obtained virus variants from five typical progressors

IC₅₀s of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC₅₀s. Shaded areas indicate the time points where autologous neutralizing activity was detected. The fifth time point in patients H1 to H4 is close to the moment of clinical AIDS diagnosis; for patient H5 the fourth time point is closest to AIDS diagnosis. Longitudinal changes in neutralization sensitivity were assessed using a Kruskal-Wallis test. * Mann-Whitney U test.
were not successful. In a previous study, we determined that individuals H1, H2, and H3 developed high-titer autologous neutralizing activity, which decreased in potency during chronic infection, whereas autologous neutralizing activity was not detected in serum from patients H4 and H5. Neutralization sensitivity for the broadly neutralizing monoclonal antibodies was assessed in a PBMC-based assay, using p24 production as a read-out for virus replication. As HIVIg contains anti-p24 antibodies which interfere with the detection of p24, we used a TZM-bl-based assay for measuring sensitivity to neutralization by HIVIg.

**Sensitivity of longitudinally isolated primary HIV-1 to HIVIg neutralization**

The majority of viruses from all timepoints and from all patients were resistant to neutralization by HIVIg, even at the highest concentration tested (500 μg/ml; Figure 8.1). Virus variants from the earliest time points from patients H2, H3, and H5 were more sensitive to HIVIg neutralization than viruses from the later time points (P = 0.047, P = 0.010 and P = 0.018, respectively), indicating that the virus populations in these patients became more resistant to antibody neutralization during the course of infection. In patients H2 and H3, changes in sensitivity for HIVIg coincided with the presence of autologous neutralizing activity in serum. The increasing resistance against HIVIg mediated neutralization of viruses from patient H5 also occurred during the asymptomatic period, but in the absence of detectable autologous neutralizing activity. Early virus variants of patients H1 and H4 were already relatively resistant to HIVIg neutralization and no significant alterations in sensitivity to HIVIg neutralization were observed over time in these individuals.

**Sensitivity of longitudinally isolated primary HIV-1 to b12 neutralization**

Clonal HIV-1 variants that were isolated shortly after SC from patients H3, H4, and H5 were highly sensitive to neutralization by b12 (IC$_{50}$ values of individual viruses varied between 0.33 and 2.91 μg/ml; Figure 8.1). In these three patients, the median IC$_{50}$ per time point for b12 increased during the course of infection (P = 0.021, P = 0.026, and P = 0.001, respectively). Moreover, at least 50% of the virus variants that were isolated at the latest time point from PBMC from each of these three individual were resistant to b12 neutralization, even at the highest concentration tested (25 μg/ml). In patient H2, a large variation in the sensitivity for b12 neutralization was observed among the earliest virus variants, ranging from 50% neutralization at 2.18 μg/ml to resistance against neutralization at 25 μg/ml b12. In contrast, viruses isolated at the midpoint of the asymptomatic phase of infection were with no exception highly sensitive to b12 neutralization. Resistance to b12 mediated neutralization increased again late in infection, as the late virus variants had significantly higher IC$_{50}$s compared to the b12 sensitive virus variants isolated during asymptomatic infection (P = 0.009). Interestingly, in these four patients, the major changes in sensitivity for b12 neutralization occurred late in infection, when autologous neutralizing activity was no longer present. In patient H1, changes in sensitivity to b12 neutralization of clonal HIV-1
variants isolated during the course of infection were not observed. At all time points, most viruses isolated from patient H1 were moderately sensitive to b12 neutralization, although coexisting HIV-1 variants that resisted b12-mediated neutralization were also present.

**Sensitivity of longitudinally isolated primary HIV-1 to 2G12 neutralization**

With the exception of the earliest viruses from patient H4 and a minority of viruses isolated from patients H2 and H3, all virus variants analyzed here were resistant to neutralization by 2G12 (Figure 8.1). Resistance to 2G12 neutralization correlated well with an envelope sequence that predicted the absence of one or more N-linked glycans that together form the 2G12 epitope. HIV-1 variants from patients H1 and H5 lacked an N at position 339 (N339X) at all time points that were analyzed in the course of infection. In patient H4, resistance to 2G12 neutralization of virus variants isolated at later time points in the course of infection coincided with the loss of either N386 or N392, whereas the absence

### Table 8.1: Average 2G12 IC₅₀ for HIV-1 variants with similar 2G12 epitope per time point and presence or absence of PNGS of the 2G12 epitope for corresponding viruses

<table>
<thead>
<tr>
<th>Mo since SC</th>
<th>Patient</th>
<th>n</th>
<th>2G12 epitope</th>
<th>Avg IC₅₀ (μg/ml)</th>
<th>Patient</th>
<th>2G12 epitope</th>
<th>Avg IC₅₀ (μg/ml)</th>
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<td>4</td>
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<td>5/5</td>
<td>- + - + +</td>
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<td>2G12 epitope</td>
<td>Avg IC₅₀ (μg/ml)</td>
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</tr>
<tr>
<td>26</td>
<td></td>
<td>3/5</td>
<td>- - + + +</td>
<td>&gt;25</td>
<td>H3</td>
<td>2</td>
<td>5/5</td>
</tr>
<tr>
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<td></td>
<td>1/5</td>
<td>- + - - +</td>
<td>&gt;25</td>
<td>1/5</td>
<td>4/5</td>
<td>- + - - +</td>
</tr>
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<td></td>
<td>3/5</td>
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<td>&gt;25</td>
<td>68</td>
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<td>4/5</td>
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<td>- + - + -</td>
<td>&gt;25</td>
<td>1/5</td>
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<td>2/4</td>
<td>- - - + -</td>
<td>&gt;25</td>
<td>1/5</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Mo, months; SC, seroconversion; Avg, average; n, number of viruses/total number of viruses tested.</th>
<th>2G12 epitope</th>
<th>Avg IC₅₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+, PNGS present; -, PNGS absent.</td>
<td></td>
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</table>
of N295, N332 and/or N339 was observed in 2G12 resistant HIV-1 variants from patient H3. 2G12 neutralization sensitive and resistant viruses co-existed in patient H2 throughout the course of infection. 2G12 resistant variants in this patient missed one or more PNGS at positions 295, 339, 386 and/or 339.

**Sensitivity of longitudinally isolated primary HIV-1 to 2F5 neutralization**

The earliest virus variants isolated from patients H2, H3, H4, and H5 were all highly sensitive to 2F5 neutralization (Figure 8.1). Over the course of infection, the majority of virus variants remained sensitive to 2F5 neutralization (Figure 8.1), although at one or more time points during infection, each of these four patients harboured a minority of viruses that resisted 2F5 neutralization even at 25 μg/ml. In viruses from patients H2, H4, and H5, sensitivity to 2F5 neutralization correlated well with the absence of mutations in the 2F5 epitope (data not shown). 2F5 neutralization sensitive viruses from patient H3 did contain a number of mutations in the core 2F5 epitope, but these did not involve the central DKW sequence (Table 8.2). In agreement with previous observations, mutations in the 2F5 epitope were also absent in a small number of viruses that completely resisted 2F5 neutralization, which may indicate that the 2F5 epitope is not equally exposed on all viruses.

Virus variants isolated early in infection from patient H1 contained a glutamine residue at position 665 in the 2F5 epitope (DQW instead of DKW), which rendered these viruses resistant to 2F5 neutralization. However, later virus variants from this patient contained a number of mutations in the 2F5 epitope (Table 8.2), which may indicate that the 2F5 epitope is not equally exposed on all viruses.

**Table 8.2: Average 2F5 IC₅₀ per time point and 2F5 epitope sequence variant of primary HIV-1 isolated at sequential time points from patients H1 and H3**

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<th>2F5 epitope; HXB2 aa 662-668</th>
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<td>&gt;25</td>
</tr>
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<td>. . . . . . W . . . SN</td>
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<td>1/2</td>
<td>. . . . . . A . . . . SN</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Mo, months; SC, seroconversion; aa, amino acid; Avg, average; n, number of viruses/total number of viruses tested.

*Essential amino acid residues in the core 2F5 epitope are indicated in bold.*
resistant to neutralization by 2F5 (Table 8.2). During asymptomatic infection, this mutation reverted to a lysine which coincided with an increased sensitivity for 2F5 (Table 8.2). The earliest virus population in patient H1 already contained a glutamine residue at position 665, indicating that the K665Q substitution was most likely present in the transmitted virus variant. Unfortunately, since the donor of patient H1 is unknown to us, we were not able to study whether this mutation may have been introduced under the selective pressure of 2F5-like antibodies in the previous host. However, we determined that sera from patient H1 from several time points during infection did not contain neutralizing activity against an HIV-2 variant engrafted with the 2F5 epitope from HIV-1 (a kind gift from G. Shaw; data

Table 8.3: 4E10 epitope sequence of primary HIV-1 variants and average 4E10 IC50 for HIV-1 variants with similar 4E10 epitope sequence per time point from typical progressors H1, H2, H3, H4, and H5

<table>
<thead>
<tr>
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Mo, months; SC, seroconversion; aa, amino acid; Avg, average; n, number of viruses/total number of viruses tested. *, Amino acid residues essential for 4E10 recognition are indicated in bold, other contact residues are underlined.15,16
not shown). These results indicate that patient H1 did not develop 2F5-like antibodies at any time during infection, suggesting that the absence of 2F5-like antibodies may have allowed the reversion of the K665Q mutation.

**Sensitivity of longitudinally isolated primary HIV-1 to 4E10 neutralization**

Virus variants from patients H2, H3, and H4 were moderately sensitive to 4E10 neutralization, while virus variants with higher levels of resistance were present at all time points in patients H1 and H5 (Figure 8.1). Increasing numbers of viruses resistant to 4E10 even at 25 μg/ml were observed during the asymptomatic and symptomatic phase of infection in patients H2 and H4, which in patient H2 coincided with the presence of autologous neutralizing activity in serum.

In all patients, HIV-1 variants with substitutions in the 4E10 epitope were observed throughout infection (Table 8.3), although none of the mutations was located at positions critical for 4E10 recognition. However, the substitutions at position 677 in the viruses from patient H5 might play a role in their relative resistance to 4E10 neutralization.

**Figure 8.2: Resistance to HIVIg, b12, 2G12, 2F5, and 4E10 of longitudinally obtained primary HIV-1**

On the left, the percentage of virus variants per time point that resisted neutralization by HIVIg (at 500 μg/ml) and the four BNAbs (at 25 μg/ml) is shown. The right part shows the percentage of virus variants that resisted neutralization by a certain number of BNAbs at 25 μg/ml. The percentage of resistant viruses per time point is color coded using increasingly darker shades of grey. At grey-shaded time points, autologous neutralizing activity was previously detected. Mo, months.
Resistance against BNAbs of longitudinally isolated primary HIV-1

To better understand the extent of resistance against BNAbs over the course of infection, we used the neutralization data as presented in Figure 8.1 to determine the percentage of neutralization resistant virus variants per time point for HIVIg (IC$_{50}$ > 500 μg/ml) and each of the four BNAbs (IC$_{50}$ > 25 μg/ml), as well as the percentage of virus variants per time point that resisted neutralization by one or more BNAbs (Figure 8.2). Overall, most virus variants analyzed in this study were resistant against one or more BNAbs, which in most cases included 2G12. As can be expected from the distinct localization of the antibody epitopes on the viral envelope, resistance to one BNAb was not predictive for sensitivity to another BNAb (data not shown).

Only at the earliest time point in patient H4, all virus variants tested sensitive to neutralization by all four BNAbs, although even most of these viruses were resistant to HIVIg. In patients H4 and H5, who both lack autologous neutralizing activity in serum, the majority of the earliest virus variants were sensitive to three or four BNAbs. However, resistance for these BNAbs developed over the course of infection, accumulating at the latest time point in a majority of virus variants that resisted neutralization by at least 3 of the BNAbs. The majority of late virus variants from patient H3 resisted b12 neutralization, while resistance to the other BNAbs did not develop. In patient H1 the relative resistance to neutralization by all BNAbs persisted over time. In general, the absence or presence of strong autologous humoral immunity in a patient was not predictive for the emergence of virus variants that resisted neutralization by b12, 2G12, 2F5, or 4E10. Moreover, BNAb resistant viruses emerged in the absence of autologous neutralizing activity in serum.

Discussion

In the search for a vaccine that is capable of eliciting cross-reactive neutralizing antibodies, research has focused on the broadest neutralizing monoclonal antibodies that have been discovered to date: b12, 2G12, 2F5, and 4E10. Although other broadly neutralizing antibody (BNAb) specificities are likely to exist, the viral epitopes of the four known BNAbs are currently the most interesting targets for vaccine design. For this reason, it is important to understand the degree of variation in susceptibility for these four BNAbs among circulating virus variants. Thus far, the BNAb sensitivities of smaller or larger panels of unrelated viruses have been comprehensively mapped. However, changes in neutralization sensitivity over the course of infection have mainly been studied in relation to the development of the autologous NAb response, while it remains to be established whether viruses also evolve during the course of infection with regard to their sensitivity for BNAb.

Here, we performed a longitudinal analysis of the resistance against neutralization by b12, 2G12, 2F5, and 4E10 among primary HIV-1 from five typical progressors. In addition, we related changes in neutralization sensitivity to mutations in the respective antibody epitopes. In general, the earliest virus populations were most sensitive to BNAb neutralization, which
might point towards the existence of a certain transmitted virus phenotype, which has
previously been observed for subtype A and C HIV-1 in relation to neutralization sensitivity
for sera of transmitting partners. The fact that we determined sensitivity to broadly
neutralizing antibodies may explain why we now confirm this observation for subtype B
HIV-1 while this was not observed in previous studies in which sensitivity to neutralization
by autologous and heterologous sera was tested. However, HIV-1 infection can also be
established by BNAb resistant viruses, as was observed in patient H1.
In patients who early in infection had viruses that were sensitive to BNAb neutralization, a
gradual increase in resistance to neutralization by one or more BNAb was observed during
later stages of disease. Most notably, virus populations changed from highly sensitive to
b12 neutralization early in infection to partially or completely resistant to b12 neutralization
in the late asymptomatic phase in three out of five patients. Previously published reports
did not show this change in b12 neutralization sensitivity during infection, which might
be explained by the fact that early and late viruses in these studies were not obtained from
the same individuals, by differences in sampling dates of earliest viruses (between 2 and
4 months after estimated SC date in our study as compared to around the moment of
seroconversion in the study by Keele et al.) 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. While
our data are discrepant from previous observations for sensitivity to b12 neutralization, the
observation that viruses from chronically infected patients in the study by Rusert et al. were
more resistant to neutralization by 2F5 and 4E10 corresponds to the increasing numbers of
(moderately) neutralization resistant viruses later in infection as observed in our present
study. With the exception of the highly 2G12 sensitive viruses in patient H4, prevalence of
2G12 resistant viruses was already relatively high early in infection, and maintained during
later stages of infection.
Antibodies b12, 2F5, and 4E10 are thought to target some of the most conserved regions
of the viral envelope. It is therefore intriguing that BNab resistant virus variants emerge
during the course of natural HIV-1 infection and this raises the question which mechanisms
are responsible for their selection. The autologous NAb response has been shown to
continuously select for escape variants which are resistant to circulating antibodies. In
patients H2, H3, and H5, the increasing resistance to HIV1g neutralization early in infection
may reflect the escape from autologous NAbs with similar epitope specificities as the NAbs
in HIV1g. However, a large proportion of the autologous neutralizing activity seems to be
directed against the variable regions of the viral envelope, making it unlikely that escape
from these antibodies would influence the sensitivity for certain BNAb, such as the MPER-
directed 2F5 and 4E10. Mutations in the V2 region have been associated with resistance
against b12, indicating that escape from antibodies targeting the V2 loop may affect
b12 sensitivity. However, the observation that b12 resistant virus variants emerged late in
infection when the autologous NAb response has subsided, argues against the selection of these variants by NAb pressure. Alternatively, viral evolution in late stage disease, driven by reducing numbers of target cells and/or the absence of strong immune responses may result in changes in the viral envelope that also affect the binding to neutralizing antibodies. In particular, adaptation to a more efficient CD4 usage at later disease stages may lead to an increased resistance against b12 as was observed in this study, as b12 competes with CD4 for attachment to the CD4 binding pocket. On the other hand, b12 sensitive virus variants (in patient H4), as well as 2F5 sensitive viruses (in patients H3, H4, and H5) persisted and coexisted with resistant variants. The continuous presence of BNAb sensitive virus variants in these patients suggests that the acquisition of BNAb resistance did not lead to a selective advantage and might have occurred randomly, rather than as a result of strong selective pressure.

As b12 recognizes a discontinuous conformational epitope, it is difficult to analyze which mutations in the viral envelope contributed to resistance against this BNAb. Mutations in various regions of the envelope have been described to influence sensitivity to b12 neutralization, although many of these changes seem to be isolate-specific. Indeed, in viruses from patient H5, we have identified a number of mutations in the envelope V1, V2 and V4 regions which appear to increase b12 resistance (Bunnik et al., manuscript in preparation), while these mutations were not found in late viruses from patients H3 and H4, who also displayed a b12 resistant phenotype.

In agreement with previous reports, a good correlation was observed between the absence of one or more of the five potential N-linked glycosylation sites that may harbour the glycans of which the 2G12 epitope is composed and the viral inhibition by 2G12. Of the 117 virus variants that lacked one or more glycans, only 4 viruses were sensitive to 2G12 neutralization. The 14 viruses that contained an intact 2G12 epitope were all highly or moderately sensitive to 2G12 neutralization. For 2F5, little sequence variation was observed in the core epitope, in line with a general sensitive neutralization phenotype of the viruses. Patient H1 was the only individual in which a substitution in the epitope (ELDQWA) of the virus evidently influenced its susceptibility to 2F5 neutralization. This K to Q substitution at position 665 has previously been described as a 2F5 escape mutation in vitro. It has been shown that resistance against 2F5 in vivo might be difficult to achieve and often results in variants with a reduced fitness. Among 309 subtype B sequences in the Los Alamos database, 3.6% harboured a K665Q substitution (data not shown), which was the most frequently observed substitution among sequences which did not contain the wild-type K residue at position 665 (13.0% of all sequences). These data indicate that the K665Q substitution might be a relatively common pathway to escape from 2F5-like antibodies, although we cannot exclude that the mutation was introduced by random sequence variation.

In this study, we have used a maximum of five clonal HIV-1 variants per time point to assess
the variation in neutralization sensitivity at that time point. Although it will be difficult to establish how representative each virus clone is in relation to the viral quasispecies, the use of clonal virus variants has clear advantages over the use of for example virus from bulk culture. The use of bulk virus will most likely underestimate the amount of variation within the quasispecies, as the neutralization sensitivity of a bulk virus population will largely be determined by the most neutralization resistant variant within that bulk. Moreover, the range in neutralization sensitivities of clonal HIV-1 variants in our study was very small at some time points, suggesting that when we do observe variation in sensitivity to Ab neutralization between clonal HIV-1 variants, this probably is a true reflection of variation in neutralization sensitivity within the viral quasispecies.

It has been suggested that in a PBMC-based assay, the presence of LPS in serum or antibody preparations may result in the suppression of HIV replication by chemokines that are released from monocyte-derived macrophages (MDM) in response to LPS 46, which can subsequently incorrectly be interpreted as antibody mediated neutralization. For our experiments, we used a single batch of pooled PBMC from 12 healthy blood donors. The observation that many viruses tested negative for neutralization by one or more of the four BNAbs suggests that our assay is not confounded by aspecific viral inhibition as a result of the presence of LPS.

In conclusion, we have shown that, with a few exceptions, the earliest virus populations isolated from five typical progressors were most sensitive for neutralization by BNAbs, and that virus resistant to one or more BNAbs developed over the course of infection in most individuals. These BNAb resistant virus variants have most likely not been selected by antibody pressure, indicating that other selective processes may be involved. For vaccine design, it will be important to understand which mechanisms drive the selection of BNAb resistant virus variants.

ACKNOWLEDGEMENTS

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REFERENCES


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 autologous neutralizing serum activity, indicating that escape from antibody neutralization may not be the only selection pressure that favors outgrowth of b12 neutralization resistant viruses.

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 5x10⁶ 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 (IC50s) were determined by linear regression.

(ii) U87-based assay: This assay was performed by Monogram Biosciences as part of a larger study 27. The preparation of pseudotyped viral particles is described in detail elsewhere 28. A recombinant virus assay involving a single round of virus infection was used to measure cross-neutralization activity of the sera 29,30. 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 31-33. Nucleotide sequences of all virus clones per individual were aligned using ClustalW in the software package of BioEdit 34, 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 TCID_{50} 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 1x10^6 cells/ml for culture. Fresh PHA-stimulated PBMC (1x10^6) in a volume of 1 ml were added at day 5 and day 8. Cultures were maintained for 11 days. Samples (75 μl) for 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.

Interferon (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 multiscan, 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 1x10^5 cells/well in triplicate. Responses were measured against an excess concentration of 10 μg/ml of the following peptides: FYKLDVVPI and mutants FYKLDIVPI and FYTDIVPI (positions 176-184), and FYCNSTQLF and mutants FFCNSTLLF, FYCNPSTLF, FYCNSKLF, and FYCNSTKLF (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/million PBMCs, after subtraction of the negative control values, were considered to have positive results.

Statistical analysis

For calculations and statistical analyses, viruses with IC_{50}s 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. 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_{50}s of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC_{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_{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 s = 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 s = 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 Env of 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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<th>Months since SC</th>
<th>Virus clone</th>
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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; IC50, 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

(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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Netherlands HIV Monitoring Foundation and are financially supported by The Netherlands National Institute for Public Health and the Environment. This study was financially supported by the Dutch AIDS fund (grant 2004064), The Netherlands Organization for Scientific research (NWO; grant 918.66.628), and the European Community’s Seventh Framework Programme NGIN (FP7/2007-2013) under grant agreement no 201433.

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b12 resistance during natural HIV-1 infection
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. IC₅₀ values of individual virus clones as determined by linear regression are shown. The horizontal lines represent the median IC₅₀ values. 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.
b12 resistance during natural HIV-1 infection
Evolution of human immunodeficiency virus type 1 in a patient with cross-reactive neutralizing activity in serum
Chapter 10

**Abstract**

Analysis of longitudinally obtained HIV-1 *env* sequences from an individual with reported cross-reactive neutralizing activity revealed that the majority of viral variants obtained from serum between 4 and 7 years after seroconversion were unable to persist in peripheral blood. Here we show that these viral variants were more sensitive to autologous serum neutralization, had shorter envelopes with fewer potential N-linked glycosylation sites, and showed lower replication kinetics than successfully evolving HIV-1 variants. These data reflect the host selection pressures on phenotypic characteristics of HIV-1 and illustrate in detail the dynamic interaction between HIV-1 and its hosts’ humoral immune responses.
The high mutation rate of HIV-1, which is the result of rapid replication dynamics in combination with an error-prone HIV-1 reverse transcriptase and a lack of proofreading, contributes to the high genetic variability of the virus and results in the continuous emergence of new viral variants. The generation of a genetically diverse viral population allows HIV-1 to adapt to the host environment by facilitating the escape from the host immune responses and the selection of viral biological properties such as co-receptor use and replication capacity. The envelope glycoprotein (Env) of HIV-1 is highly variable, as reflected by a sequence variability which may be as high as 10% within the viral population in a single individual. The random generation of single point mutations in the viral envelope gene, together with insertions and/or deletions, facilitates escape from neutralizing antibodies by altering or shielding the antibody epitope. Viral escape variants are rapidly selected due to the humoral immune pressure eliminating the neutralization sensitive virus variants, and thereby changing the genetic composition of the viral population.

Recently, we reported on the comparison of longitudinally obtained HIV-1 envelope glycoprotein sequences from viral RNA in serum (serum-RNA), replication competent clonal HIV-1 variants (CV) isolated from Peripheral Blood Mononuclear Cells (PBMC) and proviral DNA from PBMC (PBMC-DNA) from the same HIV-1 infected individual. Interestingly, in one of the four patients studied, the viral population evolved in two separate lineages: viral population 1 (VP-1) and viral population 2 (VP-2). This has been observed previously for co-existing CCR5- (R5) and CXCR4-using HIV-1 variants. In our currently studied patient, R5 variants were present in both lineages, but CXCR4-using variants were only found in VP-2. VP-1 was constituted by the majority of the viral serum-RNA sequences from the first two time points studied and two PBMC-DNA sequences from the third time-point, lacked progeny at later stages of the infection, suggesting negative selection of those viral variants. VP-2, initially mainly made up of viral sequences obtained from PBMC, did lead to progeny at later time points, both in serum and PBMC (Figure 10.1A). This individual had a typical clinical course of infection (Figure 10.1B). To understand the mechanisms contributing to the negative selection of the majority of the viral population present in serum in the period between year 4 and 7 after seroconversion (SC), we compared molecular and phenotypic properties of the initially co-existing HIV-1 populations that did or did not successfully generate progeny virus that persisted in peripheral blood.

From longitudinally obtained blood samples (9 years of seropositive follow-up, 4 different time points; figure 10.1B), a total of 29 gp160 envelope (env) sequences were generated from serum-RNA, 37 env sequences from PBMC-DNA, and 19 env sequences from CV as described previously. Genbank numbers GU455456-GU455475 and HQ231027-HQ231090.
Figure 10.1: Maximum-likelihood tree of gp160 env sequences from viral RNA in serum, PBMC proviral DNA and clonal HIV-1 variants, and clinical parameters

(A) ML tree was rooted using the root that maximized the correlation of root-to-tip divergence as a function of sampling time. Bootstrap support with values >70% are shown. The scale bar (horizontal line) indicates branch length corresponding to 0.01 substitutions per site. Sequences used to clone NL4-3/Env chimeras are circled and the names are given. The two viral populations are indicated. Figure is edited from Edo-Matas et al. 26. (B) The CD4+ T-cell counts are shown in black with the legend on the left y-axis, while viral RNA load data are indicated in gray with the legend on the right y-axis. Time of AIDS diagnosis is indicated by an arrow, sampling time points are indicated with a dot. Figure is edited from van Gils et al. 7.
Figure 10.2: The molecular env characteristics of VP-1 and VP-2

(A) Sequence logos depicting the amino acid conservation pattern across a multiple alignment of the first and second variable loops (V1V2) of all sequences of VP-1 and all sequences of VP-2. The height of the letter indicates the degree of conservation of the most common amino acid at that position. Weblogo (http://weblogo.berkeley.edu/) was used to create the sequence logos. * indicates a potential N-linked glycosylation site. (B) Longitudinal analysis of changes in number of PNGS and length of gp160 and the V1V2 region of viral variants from VP-1 and VP-2. Each dot represents one virus variant, grey dots represent X4 variants. The horizontal bars indicate average values per time point and P-values were calculated using a nonparametric t-test for independent samples. * indicates that P-value is not significant when R5 and X4 variants are analyzed separately. AA, amino acid.
found mainly, although not exclusively, in the first and second variable loops (V1V2) (Figure 10.2A) and the third constant region of env. Some of those mutations altered the number of potential N-linked glycosylation sites (PNGS) resulting in a significantly higher number of PNGS in viruses from VP-2 than in VP-1, in particular in the V1V2 region (Figure 10.2B). Additionally, the gp160 env of VP-1 viruses was significantly shorter than the gp160 env from VP-2 viruses. Within VP-2, CXCR4-using (X4) variants had longer gp160 envs than CCR5-using (R5) variants. However, even when the analysis was restricted to R5 variants, the gp160 env genes in VP-2 viruses were still longer, albeit only statistically significant at the 83 month time-point (Figure 10.2B). Increases in envelope length and number of PNGS have previously been described to decrease the neutralization sensitivity of HIV-1. Therefore we tested the sensitivity of several virus variants from both VP-1 and VP-2 to neutralization by four broadly neutralizing antibodies and autologous serum. HIV-1 gp160 env sequences obtained from serum-RNA at 47 (n=2), 68 (n=2), and 83 (n=1) months post-SC, and from CV at 47 (n=2), 68 (n=2), 83 (n=2) and 107 (n=1) months post-SC were cloned into the viral backbone NL4-3Δenv to create replication competent chimeric viruses (NL4-3/Env chimeras) through recombination, as described previously. The NL4-3/Env chimeras were tested for their neutralization sensitivity by three-fold serial dilutions of broadly neutralizing antibodies (BrNAbs) b12, 2G12, 2F5 and 4E10, with a starting concentration of 25µg/ml in triplicate, and two-fold serial dilutions of autologous serum obtained at 68 and 83 months post-SC, with a starting dilution of 1:50 in triplicate. The NL4-3/Env chimeras with the gp160 env from VP-1 viruses (VP-1-chimeras) showed similar sensitivity to neutralization by the four BrNAbs tested as compared to the NL4-3/

Table 10.1: Neutralizing sensitivity of NL4-3/Env chimeras from VP-1 and VP-2

<table>
<thead>
<tr>
<th>Months since SC</th>
<th>NL4-3/Env chimeras</th>
<th>IC₅₀ values µg/ml</th>
<th>1/serum dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4E10</td>
<td>2F5</td>
</tr>
<tr>
<td>47</td>
<td>47_S1 VP-1</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td></td>
<td>47_S2 VP-1</td>
<td>2.05</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>47_C1 VP-2</td>
<td>0.89</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>47_C2 VP-2</td>
<td>16.74</td>
<td>3.15</td>
</tr>
<tr>
<td>68</td>
<td>68_S1 VP-1</td>
<td>19.63</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>68_S2 VP-2</td>
<td>1.80</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>68_C1 VP-2</td>
<td>&gt;25</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>68_C2 VP-2</td>
<td>5.74</td>
<td>0.37</td>
</tr>
<tr>
<td>83</td>
<td>83_S1 VP-2</td>
<td>10.16</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>83_C1 VP-2</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td></td>
<td>83_C2 VP-2</td>
<td>&gt;25</td>
<td>1.07</td>
</tr>
<tr>
<td>107</td>
<td>107_C1 VP-2</td>
<td>&gt;25</td>
<td>1.85</td>
</tr>
</tbody>
</table>

C = NL4-3/Env chimera with env from clonal HIV-1 variant
S = NL4-3/Env chimera with env from viral RNA from serum
IC₅₀, 50% inhibitory concentration
In bold, IC₅₀ more than three-times the negative control
Env chimeras with the gp160 env from VP-2 (VP-2-chimeras) (Table 10.1). However, VP-1-chimeras showed a higher sensitivity to neutralization by autologous serum than VP-2-chimeras (Table 10.1). The VP-1- and VP-2-chimeras from the earliest time-point showed higher sensitivity to autologous neutralization than the VP-1- and VP-2-chimeras from later time points, respectively, suggesting escape of both virus populations from the autologous neutralizing antibody response. There was no difference in neutralization sensitivity to the BrNAbs and autologous serum between the R5 and X4 variants. The difference in sensitivity to autologous but not to heterologous neutralization between VP-1 and VP-2 suggests that viruses of VP-1 are not more sensitive to antibody neutralization in general, but only to the neutralizing activity in autologous serum. This implies that the neutralizing antibody response of this patient may have played a role in the negative selection of those viral variants.

Serum from this individual was previously demonstrated to have cross-reactive neutralizing activity (i.e.: capable of neutralizing HIV-1 variants from different clades), which was already present 23 months post-SC. Cross-reactive neutralizing activity is considered to be directed against epitopes that are conserved amongst HIV-1 variants from different clades, and are therefore probably essential for the virus. Given that the higher sensitivity to autologous serum neutralization of the viral variants that did not persist in peripheral blood (VP-1) coincided with shorter env V1V2 regions with less PNGS, cross-reactive neutralizing activity seems to select for virus variants with longer variable loops that carry more glycans, which supports the occlusion of targeted epitopes in the conserved regions as a mechanism of viral escape. The epitope specificities for the cross-reactive neutralizing activity in this patient are currently being studied, which may help to elucidate whether viral escape also occurs by mutations of specific residues in the conserved epitopes themselves.

![Figure 10.3: In vitro replication kinetics of NL4-3/Env chimeras from VP-1 and VP-2](image)

Replication rates of 1 or 2 NL4-3/Env chimeras per time point per population are expressed as the p24 production during the logarithmic expansion after infection of PHA-stimulated PBMC. The replication capacity was tested with 100 50% tissue culture infective doses (TCID_{50}) per virus. Similar relative differences between chimeric viruses were observed when an inoculum of 500 TCID_{50} was used (data not shown). S, NL4-3/Env chimera with env from viral RNA from serum. C, NL4-3/Env chimera with env from clonal HIV-1 variant.
Next we analyzed the replication kinetics of the VP-1- and VP-2-chimeras in a PBMC based replication assay, as described previously. Interestingly, VP-1-chimeras showed lower replication kinetics as compared to VP-2-chimeras (Figure 10.3). This could suggest that the combination of lower replication kinetics and higher sensitivity to autologous neutralizing activity may have been detrimental for the persistence of VP-1. The lower replication kinetics of VP-1 may have prevented those viruses from acquiring the mutations that would have allowed the escape from the broad neutralizing humoral immune response of this patient. Alternatively or in addition, mutations required for the escape from neutralizing humoral immunity in the background of VP-1 virus variants may have come at a larger fitness cost for the virus, resulting in a viral population with lower replicating capacity, which consequently was outcompeted by VP-2.

The half life of HIV-1 in plasma is about 1.3 hours indicating that the virions that were present in serum must have been produced shortly before we detected them. We failed to detect, however, the cells that produced some of those viruses in vivo as VP-1 viruses were not represented in CV and were only in low abundance in PBMC-DNA, suggesting that the cellular source for VP-1 was outside the peripheral blood compartment. The sensitivity of VP-1 viruses to autologous neutralizing antibodies may have contributed to their vulnerability in the cell-free state and may have interfered with their infection of PBMC, halting their survival in peripheral blood. The persistence of these viruses, even throughout the third time-point analyzed, suggests that they were indeed continuously produced, probably spreading through cell-to-cell transmission outside the peripheral blood compartment to avoid the cell-free state. However, we did not detect any VP-1 virus variants after 83 months post-SC. This patient developed AIDS with CD4+ T-cell counts below 200 cells/ml at 84 months post-SC (figure 10.1B). At this stage of disease, target cell availability may become limiting and viral properties such as replication capacity may exert an even greater impact on viral survival. The inability of VP-1 viral variants to compete with the more fit VP-2 viruses for limited target cells may explain their inability to persist in peripheral blood after progression to AIDS.

In summary, analysis of longitudinally obtained HIV-1 gp160 env sequences from a single individual with reported cross-reactive neutralizing activity in serum revealed that HIV-1 variants that were unable to persist in peripheral blood were more sensitive to autologous serum neutralization, had shorter envelope glycoproteins with fewer potential N-linked glycosylation sites and showed lower replication kinetics than successfully evolving HIV-1. This suggests a role for neutralizing antibody pressure on the negative selection of those viral variants. Our observations may not be unique, but merely a reflection of the common adaptation of HIV-1 in response to the host selective pressures. The detailed collection of patient materials may have provided us with the unique opportunity to study in detail the phenotypic characteristics of HIV-1 in interplay with its host humoral immune environment.
ACKNOWLEDGEMENTS

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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.
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 en 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 immunosorbotent assay. The presence of the correct env in NL4-3 was confirmed by sequencing.

PBMC-based antibody neutralization assay

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 5x10^6/ml. After inoculation, the cells (1x10^6/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, 1x10^5 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\textsubscript{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. 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.

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.
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.

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>IC_{50} (1/serum dilution)</th>
<th>IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autologous serum</td>
<td>HIV-negative</td>
</tr>
<tr>
<td></td>
<td>77 mo post-SC</td>
<td>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; IC_{50}, 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&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>Length (aa)</th>
<th>PNGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HIV1g</td>
<td>VRC01</td>
<td>b12</td>
</tr>
<tr>
<td>H19999</td>
<td>1985</td>
<td>831.3</td>
<td>0.121</td>
<td>1.12</td>
</tr>
<tr>
<td>H18766</td>
<td>1988</td>
<td>&gt;1500</td>
<td>0.094</td>
<td>&gt;25</td>
</tr>
<tr>
<td>H19768</td>
<td>1986</td>
<td>805.4</td>
<td>0.099</td>
<td>1.46</td>
</tr>
<tr>
<td>H19342</td>
<td>1986</td>
<td>846.3</td>
<td>0.090</td>
<td>1.17</td>
</tr>
<tr>
<td>H19542</td>
<td>1985</td>
<td>632.9</td>
<td>0.524</td>
<td>1.41</td>
</tr>
<tr>
<td>P197</td>
<td>2005</td>
<td>1009.0</td>
<td>0.482</td>
<td>&gt;25</td>
</tr>
<tr>
<td>P180</td>
<td>2004</td>
<td>&gt;1500</td>
<td>0.753</td>
<td>&gt;25</td>
</tr>
<tr>
<td>P004</td>
<td>2003</td>
<td>&gt;1500</td>
<td>0.345</td>
<td>&gt;25</td>
</tr>
<tr>
<td>P002</td>
<td>2003</td>
<td>1316.3</td>
<td>&gt;5</td>
<td>5.30</td>
</tr>
<tr>
<td>P127</td>
<td>2005</td>
<td>&gt;1500</td>
<td>&lt;0.078</td>
<td>0.21</td>
</tr>
</tbody>
</table>

SC, seroconversion; IC<sub>50</sub>, 50% inhibitory concentration; aa, amino acid; PNGS, potential N-linked glycosylation site.

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
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 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 Env 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 suggest 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{23,51,52}. 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**

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collection and analysis, decision to publish, or preparation of the manuscript. We thank J. Alcami (Instituto de Salude 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.

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Appendix Table A1.1: Primer combinations for amplification of separate PCR products

<table>
<thead>
<tr>
<th>Region</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start to V1</td>
<td>5’-TCCTAGGATTGGTGCTCCATGGCTT-3’</td>
<td>3’-GCAATTTAAGTAACACAGAGGTGG-5’</td>
</tr>
<tr>
<td>V1 to stop</td>
<td>5’-GCCTCTTCAATATCACCACACAGG-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGGCTTGGTGCT-5’</td>
</tr>
<tr>
<td>V2 to stop</td>
<td>5’-CACCTCAGTCATTACACAGGC-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGGCTTGGTGCT-5’</td>
</tr>
<tr>
<td>V1V2</td>
<td>5’-GGATCCAAAGGCTAAAGCCATGTG-3’</td>
<td>3’-GCCGGGGCACAATAATGTATG-5’</td>
</tr>
<tr>
<td>V3 to stop</td>
<td>5’-ATGAGACAAGCACATTTGTAAC-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGGCTTGGTGCT-5’</td>
</tr>
<tr>
<td>V3</td>
<td>5’-CTGCCAATTTCTCGGACAATGC-3’</td>
<td>3’-GTAATCCTAATGTTACAGTGTCG-5’</td>
</tr>
<tr>
<td>Start to V4</td>
<td>5’-TCCTAGGATTGGTGCTCCATGGCTT-3’</td>
<td>3’-ATCACAGTAGAAAATTTCCCGC-5’</td>
</tr>
<tr>
<td>V4 to stop</td>
<td>5’-CACACTTCAATGACAGAAATAA-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGGCTTGGTGCT-5’</td>
</tr>
<tr>
<td>gp120</td>
<td>5’-TCCTAGGATTGGTGCTCCATGGCTT-3’</td>
<td>3’-CCATAGTGGCTTGGCTGCT-5’</td>
</tr>
<tr>
<td>gp41</td>
<td>5’-ACAAAGGCGAAAGAGAAGAGTG-3’</td>
<td>3’-CTGCCAATCAGGGAAGTAGGCTTGGTGCT-5’</td>
</tr>
</tbody>
</table>
Chapter 12

General discussion
**Protection against pathogenic infections**

The twentieth century witnessed the introduction of several successful vaccines, including those against diphtheria, measles, tetanus, yellow fever, hepatitis A and B, influenza, smallpox and polio. As vaccines became more common, many people began to take them for granted. However, vaccines remain elusive for many important diseases, including malaria and HIV-1 infection.

For each of the known vaccines, protection has been achieved by mimicking infection with the pathogen and thereby establishing immunologic memory that can rapidly respond should an actual infection occur. This has been achieved with the use of live attenuated viruses, killed viruses or recombinant viral proteins which all do not cause the disease but which do elicit strong and long-lasting immune responses.

The tremendous global success with viral vaccines raises the question as to why HIV-1 vaccine development is so difficult. Many of the difficulties lie in distinct properties of this virus compared with other viruses. Foremost among these is the enormous sequence diversity of HIV-1, which can be as high as 35% between viruses from different subtypes and the relative inaccessibility of the conserved epitopes. Moreover, the lack of understanding of the immune responses that can control HIV-1 replication, for instance in elite controllers and high risk seronegative individuals, makes the development of a protective vaccine even more challenging.

It is assumed that a protective vaccine should elicit cross-reactive neutralizing humoral immunity in combination with a cellular immune response. In combination, these responses ideally can protect against acquisition of infection or second best, against disease progression by reducing viral load which will also have an impact on the spread of HIV-1 in the population.

The studies described in this thesis are focused on cross-reactive neutralizing humoral immunity and the interactions between HIV-1 and its host humoral immune responses. In this chapter the implications of the results described in this thesis will be discussed in view of the possibilities for vaccine immunogen design and to elicit sterilizing immunity against HIV-1.

**Humoral immunity in HIV-1 infection**

The majority of HIV-1-infected individuals mount an HIV-1-specific neutralizing humoral immune response within weeks to months after primary infection. This response is considered to be strain-specific as neutralizing activity is generally restricted to the autologous virus variant and mainly directed against the variable regions of the envelope glycoprotein. Longitudinal studies have shown that HIV-1 rapidly and repeatedly escapes the neutralizing antibody response mounted during HIV-1 infection. The presence of neutralizing antibodies is a burden to the virus as it drives the continuous evolution of the HIV-1 envelope glycoprotein. As a consequence of this selection, the majority of
the virus population in an infected individual is only weakly, if at all, neutralized by the contemporaneous antibody repertoire 17,21,24.

With time, as the virus population diversifies and the immune response matures, neutralization can also be detected against heterologous HIV-1 variants16,17,19,21. In chapter 2 we observed that the neutralizing activity in sera of participants from the Amsterdam Cohort Studies who are chronically infected with HIV-1 subtype B was preferentially directed against the subtype B HIV-1 variants in a multi-subtype virus panel that also included clade A, C, and D HIV-1 variants. Subtype-specific humoral immunity may provide new leads on the way to a potent HIV-1 vaccine. However, developing and administering multiple HIV-1 vaccines is far less ideal than having a single vaccine that would cover all circulating HIV-1 variants.

During the first three years of infection approximately 30% of HIV-1 infected individuals in the Amsterdam Cohort Studies developed cross-reactive neutralizing activity in serum (chapter 4), with the ability to neutralize viruses from different subtypes 27. In this same cohort one so called “elite neutralizer” was identified with a HIV-1 specific neutralizing activity in serum with tremendous potency and breadth 28. The prevalence of individuals with cross-reactive and elite neutralizing activity in the Amsterdam Cohort is in agreement with that observed in several other cohorts in other geographic regions 28-30. The relatively high prevalence of cross-reactive neutralizing activity suggests that the epitopes that are capable of eliciting these humoral responses are accessible and immunogenic on the native gp160 spike of HIV-1. Moreover, the fact that truly broadly neutralizing antibodies exist, implicates that a single protective antibody-based vaccine against HIV-1 may be an achievable goal. The sequence variation in the HIV-1 envelope glycoprotein may thus be less problematic for the choice of epitope specificities that a vaccine should cover. Indeed, it may not so much be a matter of whether an epitope is present but rather if it is accessible on HIV-1 variants from different subtypes. The fact that recently identified cross-reactive neutralizing antibodies PG9, PG16 and VRC01 have neutralizing activity against the majority of primary HIV-1 variants 31,32, suggests that the epitopes of at least these neutralizing antibodies are indeed accessible predicting the potential success of a vaccine that would be capable of eliciting this type of antibodies.

**Factors associated with the development of cross-reactive neutralizing humoral immunity**

To support HIV-1 vaccine development, more insight is needed into the host and viral factors that are associated with the ability of the host to elicit a cross-reactive neutralizing humoral immune response, and how such a neutralizing serum response evolves over time. The development of a potent cross-reactive neutralizing humoral immune response takes at least 2 to 3 years (chapter 3). It has been shown that the breadth of neutralization is correlated with time since infection 33. This time may be required for the affinity maturation during which the neutralizing antibodies gain affinity and become highly potent 34-36.
We have also observed that the ability of serum to neutralize different viruses is directly related to the neutralization titer in serum (chapter 2). This may imply that sera with highly cross-reactive neutralizing ability in general harbor multiple epitope specificities or that a high quantity of a single antibody specificity is more potent, even against unrelated HIV-1 variants. It may also be that the development of broadly neutralizing antibodies is related to the evolution of HIV-1. As discussed above, as neutralizing antibodies emerge during the course of infection, they will rapidly select for HIV-1 escape variants that have mutations in the epitope that is recognized by these antibodies. In turn, these viral escape variants may contribute to the affinity maturation of the neutralizing antibody response. By continuous cycles of selection for escape variants that subsequently drive affinity maturation, antibodies with higher potency and breadth may emerge. This may imply that high concentrations of cross-reactive neutralizing antibodies will increase the chance that such an antibody can bind, for instance when the epitope is only transiently accessible in the trimeric structure. An alternative hypothesis is that instead of affinity maturation of the original antibody response, the constantly emerging escape variants continuously elicit novel antibody responses during the course of infection which in combination may provide a serum with a cross-reactive neutralizing phenotype. Indeed, recent studies have demonstrated that the individual epitope specificities did not account for the breadth of neutralizing activity in serum whereas the combination of these different antibodies did approach the neutralization phenotype of the patient serum.

In chapter 3 we observed that a high plasma viral RNA load set-point and low CD4+ cell count set-point were both associated with the development of cross-reactive neutralizing activity. Furthermore, we observed that higher cross-reactive neutralizing activity was significantly associated with lower CD4+ T cell counts already before and soon after infection (chapter 4). In a model for Lymphocytic Choriomeningitis Virus (LCMV) infection, a reduction in CD4+ T cell numbers prior to infection reduced polyclonal B cell stimulation and enhanced protective antibody responses in terms of earlier onset and higher titers without impairing protective CD8+ T cell responses. The correlation between the development of cross-reactive neutralizing activity and a high plasma viral RNA load indicates that the development of potently neutralizing humoral immunity apparently requires exposure to a sufficient amount of antigen. Indeed the prevalence of cross-reactive neutralizing activity in serum from elite controllers and viremic controllers was much lower as compared to typical progressors.

A certain level of antigen is apparently required to drive the humoral immune response. However not all infected individuals with high viral loads develop cross-reactive neutralizing antibodies. This might relate to differences in the accessible of certain epitopes in the trimeric HIV-1 envelope structure. It can be hypothesized that a transmitted virus with more exposed conserved epitopes might elicit neutralizing antibodies with a larger breadth. Viruses with longer variable loops 1 and 2 and more potential N-linked glycosylation...
sites might not elicit such antibodies as the CD4-binding site may be less accessible on these viruses as we observed in chapter 11.

To be more conclusive on the factors that determine the development of cross-reactive neutralizing humoral immunity, the neutralizing component in serum needs to be identified. This will show whether the breadth of the neutralizing activity in serum is determined by a single high affinity antibody directed against a highly conserved epitope in the envelope glycoprotein, or if it is the combined effect of multiple co-existing neutralizing antibodies directed at multiple distinct regions of the envelope. It cannot be excluded that both scenarios exist and that the number of antibody specificities in cross-reactive neutralizing sera may vary between individuals.

**Effect of cross-reactive neutralizing humoral immunity on HIV-1 disease**

It remains to be established how HIV-1 neutralizing activity in vitro relates to protection from infection in vivo. In non-human primate studies, passive transfer of broadly neutralizing antibodies completely blocked infection by a chimeric simian-human immodeficiency virus, while in humans, passive transfer of broadly neutralizing antibodies delayed HIV-1 rebound after cessation of antiretroviral therapy.

Previous studies have shown that autologous strain specific neutralizing activity does not contribute significantly to the control of HIV-1 infection. In chapter 4 we analyzed the AIDS free survival time of individuals with strong, moderate or absent cross-reactive neutralizing activity, and showed that cross-reactive neutralizing activity in serum did not have an impact on the clinical course of HIV-1 infection. Moreover a similar prevalence of cross-reactive neutralizing serum activity in long-term non-progressors (LTNP) and progressors at 2 and 4 years post-SC was observed (chapter 4). The absent correlation between the presence of cross-reactive neutralizing immunity and disease progression could point towards a fading humoral immunity in the progressive course of infection. Previous studies have shown that the autologous neutralizing antibody response decreases over time, probably as a result of the depletion of CD4+ T-cell help during chronic infection. In addition, vaccination of HIV-1 infected individuals against other pathogens showed reduced immune responses. In the longitudinal analysis that is described in chapter 7, cross-reactive neutralizing humoral immunity was preserved in both LTNP and progressors, even after the moment of AIDS diagnosis. In contrast, autologous neutralizing activity was only observed against viruses that were isolated early in infection. Moreover, the limited autologous neutralizing activity against early viruses was lost after AIDS diagnosis. The absent association between cross-reactive neutralizing immunity and the clinical course of HIV-1 infection together with the limited autologous neutralizing activity might also be suggestive of rapid viral escape from cross-reactive neutralizing humoral immune pressure, despite the fact that cross-reactive neutralizing antibodies are considered to be
directed against conserved epitopes. We indeed observed that HIV-1 can rapidly escape from autologous humoral immunity with cross-reactive neutralizing activity together with the inability of the infected host to generate novel neutralizing antibody specificities against these escape variants (chapter 7). Furthermore, these escape mutations do not come at a fitness cost to the virus, as has been described for certain escape mutations in conserved epitopes for cytotoxic T lymphocytes. Overall, the similar potency of humoral immunity, the similar dynamics of viral escape, and the absent impact of escape on the replication kinetics of viruses from both LTNP and progressors argue against a role for neutralizing antibodies in the clinical course of infection. Possibly HIV-1 cellular immunity and host genetic background, rather than neutralizing antibodies may contribute to the control of already established infections while neutralizing antibodies may be essential for protection from infection.

**THE ADAPTATION OF HIV-1 TO HUMORAL IMMUNITY**

As previously mentioned, neutralizing antibodies rapidly select for escape variants of HIV-1 that have become resistant to neutralization. Escape from neutralizing antibodies may be mediated by mutations in the epitope as a consequence of which the antibody is no longer able to bind, or by changes in other regions of the envelope that prevent access of the antibody to the neutralizing epitope.

In chapter 7 we observed that the escape of HIV-1 to cross-reactive neutralizing humoral immunity was correlated with an increase in length of the viral envelope glycoprotein (Env) and the number of potential N-linked glycosylation sites (PNGS) in Env. Positive selection pressure was observed in the variable regions in Env, suggesting that escape is not mediated by mutations in the conserved epitopes but rather by changes in the variable regions that then prevent access of the neutralizing antibodies to their target epitopes. This also explains why escape from cross-reactive neutralizing humoral immunity does not coincide with a reduced replication fitness of the virus (chapter 7). Interestingly, the exchange of the different variable regions from a neutralization sensitive into a neutralization resistant HIV-1 variant that were obtained respectively early and late in infection from a single individual, revealed that the V1V2 region is indeed a strong determinant for neutralization sensitivity (chapter 11). This was confirmed by the observation that increasing neutralization sensitivity coincided with shorter V1V2 loops and fewer PNGS in tier categorized neutralization sensitive and resistant HIV-1 variants (chapter 11).

The adaptation of HIV-1 at a population level to neutralizing humoral immunity also coincided with an increased length of Env and number of PNGS in Env, mainly concentrated in the V1 region. Moreover, exchange of the V1V2 regions from neutralization sensitive HIV-1 variants from historical seroconverters with V1V2 regions from neutralization resistant HIV-1 variants from contemporary seroconverters could decrease the neutralization sensitivity (chapter 11). These findings, together with studies from others, demonstrate
that the increase in length and number of PNGS of the Env V1V2 region of the HIV-1 envelope glycoprotein 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.

In addition to the changes in the Env V1V2 region also other changes in the envelope glycoprotein may influence neutralization resistance. It has been shown that mutations outside an epitope may influence the conformational structure of the envelope and thereby the exposure of an epitope. In chapters 8 and 9 we observed that over the course of infection in a substantial proportion of HIV-1-infected individuals viruses emerged that were resistant to one or more broadly neutralizing antibodies while the patients from whom these viruses were isolated lacked HIV-1 specific humoral and cellular immunity. For vaccine design, it will be important to understand which mechanisms may drive the selection of neutralization resistant virus variants.

In chapter 6 we studied in detail the HIV-1 evolution in several patients using different viral sources to better understand the selective pressure of humoral immunity on HIV-1 evolution. We observed that clonal HIV-1 variants isolated from PBMC may equally represent the viral quasispecies in blood as sequences obtained from serum and PBMC proviral DNA. However, certain selective forces, such as neutralizing humoral immunity, may drive differential evolution of the cell-free and cell-associated virus pool, reflected in separate clusters of HIV-1 sequences that were obtained from serum RNA in some patients at certain time points. In chapter 10 it was shown that the serum HIV-1 variants that were unable to persist in peripheral blood were more sensitive to autologous serum neutralization and had shorter Env with fewer potential N-linked glycosylation sites than successfully evolving HIV-1 variants, suggestive of a role for neutralizing antibody pressure on HIV-1 evolution.

**Directions for HIV-1 vaccine development**

The nature of neutralizing antibody responses in natural HIV-1 infection may offer new clues for vaccine design. Recently, the extremely potent and broadly neutralizing antibodies VRC01, and PG9 and PG16 were identified, which all seem to target conserved regions of the envelope glycoprotein. One of the current approaches is to use the epitopes of very potent broadly neutralizing antibodies as immunogens to elicit HIV-1 specific neutralizing antibodies with similar potency and breadth. The epitopes targeted by the currently known broadly neutralizing antibodies are the conserved domains on the envelope trimer, located at the CD4-binding site (VRC01 and b12), glycan shield (2G12), conserved regions of the V1, V2 and V3 region (PG9 and PG16), and the membrane proximal external region (MPER) of gp41 (2F5 and 4E10). The fact that the majority of primary HIV-1 variants are neutralized by one or more of the currently known broadly neutralizing antibodies, implies that the epitopes for these
broadly neutralizing antibodies are accessible on primary viruses. While it is not precisely known what level of Abs are required for protection against HIV-1 infection, recent work examining the efficacy of low antibody titers against low dose repeated pathogenic simian-human immunodeficiency virus challenge in macaques indicates that high concentrations of antibodies may not be needed to provide protective benefit. The individual identified as elite neutralizer in chapter 4 represents a new resource for the identification of novel monoclonal antibodies that are both broad and potent against HIV-1. Another new development is the use of a B-cell mosaic vaccine to optimize the immunogenicity in an attempt to elicit subtype-specific or even cross-clade neutralizing antibodies as described in chapter 5. A different direction of immunogen design is the use of only the epitope itself, such as the CD4-binding site. By using glycans to cover other immunogenic targets of the protein except for the desired epitope, the chance may be increased that antibodies against that particular epitope are elicited. However it should be taken into account that the immunogen or epitope that will be used to elicit an antibody response is also accessible on currently circulating primary HIV-1 variants. As illustrated in chapter 11, HIV-1 has the ability to protect the conserved epitopes by increasing length and glycosylation of the envelope glycoprotein. It is therefore important to use an immunogen that has the natural characteristics of the envelope glycoprotein, but will direct the response to conserved epitopes to get a broad and potent response.

To date, no immunogen has been able to elicit protective neutralizing immunity in animal models. In most studies on immunogenicity, animals are primed and boosted only a few times and total follow-up times are often restricted to several weeks. It is intriguing that while we know that the development of a cross-reactive potently neutralizing antibody response in HIV-1 infected humans may take several years, we still expect this same process to happen within weeks in animal models. Although in the ideal situation one would like to achieve at least some level of protection already after priming, it cannot be excluded that the affinity maturation of HIV-1 neutralizing antibodies that is probably essential to get cross-reactive neutralizing antibodies, requires a longer period of time and multiple antigen exposures also in animal models. Therefore, in addition to the development of novel immunogens, novel designs of immunization schedules may be required.

It also remains to be established in what formulation the immunogen should be delivered. Many possibilities are being developed, from soluble proteins to DNA plasmids and viral vectors, which can all be used in multiple prime-boost combinations. The type of response that needs to be elicited also depends on the delivery system. Soluble proteins will elicit only humoral immune responses, while DNA plasmids and viral vectors can elicit both humoral and cellular responses. These gene delivery systems can deliver any type of gene into a cell and get expression of the protein. Depending on the protein, the immune response will be directed into Th1 or Th2 depending on the HLA type by which presentation of the epitope is restricted.
A first modest success was obtained with a pox virus prime, gp120 protein boost vaccine regimen in the so called Thai trial (RV144). This vaccine included gag, nef, and pol and in addition monomeric envelope glycoproteins from clades B en E, which are the major circulating clades in the region where the vaccine trial was performed. The vaccine-induced protective effect was however only modest and the identification of the immune correlates of protection and the relative contribution of each vaccine component need to be elucidated. First analyses have shown that vaccinated individuals developed HIV-1 binding antibodies in serum, however no neutralizing antibodies could be detected. It cannot be excluded that other antibody functions, such as ADCC or ADVCI may play a role in the achieved protection.

Our studies only emphasized on neutralizing antibody responses with a focus on cross-reactive activity, however other antibody functions, such as ADCC or ADVCI, and cellular immunity may also play a role in HIV-1 evolution and disease course and may be worth studying in our patients in the future as well.

**Concluding remarks**

Although neutralizing antibodies may not be able to influence HIV-1 disease course, neutralizing antibodies do have an impact on HIV-1 evolution. New insights in these interactions have revealed the importance of the accessibility of the vulnerable epitopes on the HIV-1 envelope glycoprotein in a vaccine immunogen. The fact that HIV-1 rapidly escapes from even the most potent and cross-reactive neutralizing antibodies implicates that by all means, viral replication in a new host should be prevented. A vaccine therefore should elicit protection against acquisition of HIV-1.

**References**


Summary
Samenvatting
Dankwoord
Curriculum Vitae
Summary

The need for an effective vaccine to prevent the global spread of human immunodeficiency virus type 1 (HIV-1) is well recognized. It is assumed that a protective vaccine should ideally elicit cross-reactive neutralizing humoral immunity in combination with a cellular immune response. Unfortunately, to date HIV-1 envelope-based immunogens have been unable to elicit HIV-1 specific potent and cross-reactive neutralizing humoral immune responses. The design of an immunogen that is capable of eliciting neutralizing antibodies is complicated as HIV-1 has developed multiple mechanisms to evade neutralizing antibodies, including the inaccessibility of relevant epitopes due to the trimeric structure of the HIV-1 envelope protein, the density of glycosylation, and the occluding variable loops on the outer domain of the envelope spike. A better understanding of the evasive mechanisms of HIV-1 from humoral immunity and of the mechanisms driving the development of broadly neutralizing antibodies may be crucial for vaccine immunogen design.

During natural infection, the majority of HIV-1-infected individuals mount an HIV-1 specific neutralizing humoral immune response within weeks to months after primary infection. This response is considered to be strain-specific as neutralizing activity is generally restricted to the autologous virus variant and mainly directed against the variable regions of the envelope glycoprotein. These antibodies rapidly select for escape variants of HIV-1 that have become resistant to neutralization as a result of amino acid substitutions, insertions and/or deletions in the variable regions, and/or changes in the glycan shield. The rapid escape of HIV-1 from autologous type-specific neutralizing antibodies seems to be the underlying explanation for the absent correlation between autologous humoral immunity and HIV-1 disease course. Broadly neutralizing antibodies may bypass the viral defense mechanisms as they have the ability to neutralize HIV-1 variants from different subtypes. The epitopes targeted by these broadly neutralizing antibodies are the conserved domains on the envelope trimer, located at the CD4-binding site, glycan shield, conserved regions of the V1, V2 and V3 region, and the membrane proximal external region (MPER) of gp41.

In this thesis, the prevalence, development and characteristics of cross-reactive neutralizing humoral immunity in HIV-1 infected individuals was studied. In chapter 2 we uncovered the presence of subtype-specific neutralizing activity in many HIV-1 infected individuals and observed a positive correlation between the titer and breadth of neutralizing activity in patient sera. The development of a potent cross-reactive neutralizing humoral immune response takes at least 2 to 3 years (chapter 3), albeit not in all HIV-1 infected individuals. It has become apparent that about one-third of HIV-1 infected individuals develop cross-reactive neutralizing activity in serum during the first 3 years (chapter 4), suggesting that the B cell repertoire in humans should indeed be sufficient to generate potently neutralizing antibodies in response to a vaccine. However we also observed that cross-reactive neutralizing activity in serum does not seem to have an impact on the clinical course of HIV-1 infection. Possibly cytotoxic CD8+ T-cells rather than neutralizing antibodies may contribute to the
control of already established infections while neutralizing antibodies may be essential for protection from infection.

Subtype-specific humoral immunity has been suggested as an interesting alternative to deal with the huge sequence variation that is challenging HIV-1 vaccine development. As reviewed in chapter 5, subtype-specific humoral immunity may provide new leads on the way to a potent HIV-1 vaccine. However, developing and administering multiple HIV-1 vaccines is far less ideal than having a single vaccine that would cover all circulating HIV-1 variants. Moreover, the fact that truly broadly neutralizing antibodies exist, implicates that a single protective antibody-based vaccine against HIV-1 may be an achievable goal.

To further investigate the effect of cross-reactive neutralizing activity on disease progression and the adaptation of HIV-1 to the humoral immune response, we studied HIV-1 evolution in several patients using replication competent clonal HIV-1 variants. The major advantage of working with replication competent clonal HIV-1 variants is that biological properties of the virus can be studied in the context of the original genetic background and the complete viral genome, which obviously is not the case with cloned viral gene fragments from plasma in the background of a molecular HIV-1 clone. Chapter 6 shows that replication competent clonal HIV-1 variants isolated from PBMC may equally represent the viral quasispecies in blood as sequences obtained from serum and PBMC proviral DNA. By using replication competent clonal HIV-1 variants that were isolated over the course of HIV-1 infection from 6 patients, we could demonstrate a rapidly evolving resistance to neutralization by autologous sera, explaining the absent role for neutralizing antibodies, even of those with cross-reactive neutralizing activity, on the clinical course of HIV-1 infection. Despite the fact that cross-reactive neutralizing antibodies are most likely directed against epitopes that are conserved among HIV-1 variants even of different subtypes, the escape of HIV-1 did not coincide with a loss of viral fitness (chapter 7). We observed that the escape of HIV-1 to the humoral immune response was correlated with an increase in length and number of potential N-linked glycosylation sites of the envelope glycoprotein. Another example of the interaction between HIV-1 and its host is described in chapter 8. We observed that over the course of infection in most individuals viruses emerged that were resistant to one or more broadly neutralizing antibodies. For instance b12-resistant virus variants emerged late in infection in a substantial proportion of HIV-1-infected individuals and interestingly this could occur in the absence of both humoral and cellular immunity (chapter 9). For vaccine design, it will be important to understand which mechanisms drive the selection of broadly neutralizing antibody resistant virus variants.

In addition, certain selective forces may drive differential evolution of the cell-free and cell-associated virus pool, in which case, sequences from both sources would be ideally required to obtain a more complete picture of the interactions between HIV-1 and its host. In chapter 10 longitudinally obtained HIV-1 envelope glycoprotein sequences from serum RNA, PBMC proviral DNA and replication competent clonal variants from a single
individual with reported cross-reactive neutralizing activity in serum were analyzed in detail. The results suggest a role for neutralizing antibody pressure on HIV-1 evolution as HIV-1 variants that were unable to persist in peripheral blood were more sensitive to autologous serum neutralization, had shorter envelopes with fewer potential N-linked glycosylation sites and showed lower replication kinetics than successfully evolving HIV-1 variants. The adaptation of HIV-1 to neutralizing antibodies, through the increase in length and glycosylation of the envelope glycoprotein, was further demonstrated in chapter 11. The increase in length and/or glycosylation of the V1V2 region of the HIV-1 envelope glycoprotein was shown to be directly responsible for the protection of HIV-1 against gp120-directed neutralizing antibodies, possibly by shielding underlying epitopes in the envelope glycoprotein from antibody recognition.

This thesis describes the dynamic interactions between HIV-1 and its host humoral immune responses. Although neutralizing antibodies may not be able to influence HIV-1 disease course, neutralizing antibodies do have an impact on HIV-1 evolution. New insights in these interactions have revealed the importance of the accessibility of the vulnerable epitopes on the HIV-1 envelope glycoprotein in a vaccine immunogen and the need to achieve sterilizing immunity with a neutralizing antibody based vaccine.
VACCINONTWIKKELING

De ontdekking van het eerste vaccin door Edward Jenner in 1796 tegen pokken luidde een tijdperk in waarin het mogelijk werd om mensen en dieren te beschermen tegen menig ziekteverwekker. Inmiddels zijn er al veel vaccins ontwikkeld die (gedeeltelijke) bescherming bieden tegen vele infectieziekten, zoals pokken, polio, tetanus, difterie, hepatitis en griepp. Een vaccin trent het afweersysteem om, als het later in contact komt met de echte variant, adequaat te kunnen reageren zodat de ziekteverschijnselen niet of nauwelijks optreden. Ons immuunsysteem ontwikkelt namelijk antistoffen tegen een specifieke ziekteverwekker en kan daardoor direct reageren als deze het lichaam binnendringt.

De meeste vaccins bestaan uit gedode of verzwakte ziekteverwekkers, maar ook slechts delen van een ziekteverwekker kunnen worden gebruikt. Vaccins worden steeds vaker toegepast om ons te beschermen tegen ziektes en worden daardoor meer en meer vanzelfsprekend geacht. Maar voor vele ernstige ziektes is het nog steeds niet gelukt om een effectief vaccin te ontwikkelen, zoals tegen malaria en hiv infectie.

INLEIDING IN HIV EN AIDS

Het humaan immuundeficiëntie virus (hiv) is verantwoordelijk voor aids, het verworven immuundeficiëntie syndroom (aids is de afkorting van de Engelse naam: acquired immunodeficiency syndrome). Medio 2010 waren er in Nederland ruim 13 duizend hiv-geïnfecteerde patiënten bekend en wereldwijd zijn op dit moment naar schatting ongeveer 33 miljoen mensen geïnfecteerd met hiv, waarvan de meesten in Afrika ten zuiden van de Sahara leven. Hiv kan worden overgedragen tijdens seks, via bloed-bloed contact of van moeder op kind tijdens de zwangerschap, bevalling of via de moedermelk.

Het kenmerk van een virus is dat het de cellen van een ander organisme nodig heeft om zich te vermenigvuldigen. Hiv gebruikt hiervoor de CD4+ T-cellen van het menselijk afweersysteem. Deze CD4+ T-cellen zijn speciale witte bloedcellen die het afweersysteem coördineren in het geval van een infectie. Door de hiv-infectie van de CD4+ T-cellen is het virus niet alleen in staat zichzelf te vermenigvuldigen, maar schakelt het ook het beschermingsmechanisme uit waardoor het lichaam zich niet meer tegen het virus en andere ziekteverwekkers beschermt. Door de sterke vermindering van het aantal CD4+ T-cellen kunnen ook andere virussen en bacteriën, die normaal gesproken zonder problemen door het afweersysteem in de hand worden gehouden, een ziekmakende infectie veroorzaken. Het zijn in de meeste gevallen deze ‘opportunistische infecties’ op basis waarvan de diagnose aids gesteld wordt en waaraan een patiënt overlijdt. Daarnaast ontwikkelen veel aidspatiënten ook zeldzame vormen van kanker zoals het kaposisarcoom.

Het ziekteverloop van de hiv-infectie wordt bepaald door factoren die gerelateerd zijn aan zowel patiënt als virus en kan van patiënt tot patiënt sterk verschillen. Er zijn bijvoorbeeld patiënten die pas ziek worden na vijftien jaar, terwijl anderen al na enkele jaren aids ontwikkelen. Dit verschil in ziekteverloop tussen patiënten kan gedeeltelijk verklaard
worden door genetische verschillen in het afweersysteem tussen patiënten. Ook het virus zelf kan bepalend zijn voor het ziekteverloop, omdat hiv zelf ook genetisch zeer variabel is. Sinds 1996 is het mogelijk om een combinatietherapie te krijgen die er voor zorgt dat de vermenigvuldiging van hiv sterk geremd wordt. Hiv zal niet uit het lichaam verdwijnen door het gebruik van deze medicijnen. Wel kan de ontwikkeling van de ziekte aids worden geremd en kan de overdracht van het virus op iemand anders worden voorkomen.

**Neutraliserende antistoffen en hiv-vaccinontwikkeling**

Er zijn twee varianten van het aidsvirus bekend, type 1 en 2. Hiv-1 en hiv-2 veroorzaken beide aids hoewel de ontwikkeling van aids na infectie met hiv-2 vaker een langzamer verloop heeft. Beide typen zijn vermoedelijk in het begin van de twintigste eeuw van de aap op de mens overgegaan in Centraal Afrika. Hiv-1 bestaat uit verschillende groepen waarvan groep M de meest voorkomende virussoorten bevat. Groep M bestaat uit verschillende subtypen, A tot en met K, die wel tot 35% van elkaar kunnen verschillen in hun genetische materiaal. In Nederland, net als in andere westere landen, is subtype B de meest voorkomende vorm van hiv. In Zuidelijk Afrika en India is dit voornamelijk subtype C en de subtypen A en D komen voornamelijk voor in Centraal Afrika. De grote diversiteit tussen de verschillende hiv varianten over de hele wereld is een van de grote problemen bij de ontwikkeling van een vaccin tegen hiv.

Het wetenschappelijk onderzoek naar een hiv-vaccin richt zich onder andere op de ontwikkeling van een vaccin dat er voor kan zorgen dat het lichaam neutraliserende antistoffen tegen hiv maakt. Deze neutraliserende antistoffen herkennen bepaalde patronen (epitopen) op de eiwitten aan de buitenkant (envelop) van het virus. Dit envelop-eiwit wordt door hiv gebruikt om de CD4-receptor op de CD4+ T-cellen te binden om zo de cel te infecteren. De binding van neutraliserende antistoffen aan het envelop-eiwit zorgt ervoor dat het virus de cel niet meer kan infecteren. Het virus wordt zo ‘geneutraliseerd’.

In de praktijk zien we echter dat hiv gemakkelijk kan ontsnappen aan neutraliserende antistoffen. Tijdens de vermenigvuldiging van hiv ontstaan er kleine ‘foutjes’ in het genetische materiaal, waardoor de samenstelling van het envelop-eiwit kan veranderen. Hiv kan zich niet alle foutjes in het envelop-eiwit permitteren. Foutjes in bijvoorbeeld die delen waarmee het envelop-eiwit aan de CD4-receptor bindt, kunnen ervoor zorgen dat het virus de cel niet meer kan infecteren. Veranderingen in andere delen van het envelop-eiwit, de zogenaamde variabele delen, hebben geen nadelige gevolgen voor het virus en hebben bovendien als functie het afweersysteem af te leiden van de geconserveerde delen van het eiwit. Naast deze variabele delen zijn er ook een aantal suikers gebonden aan het envelop-eiwit. Suikers worden niet door antistoffen herkend, omdat ze als lichaamseigen worden gezien, waardoor het afweersysteem wordt omzeild. De suikers kunnen veranderen in aantal en positie op het eiwit om ook op deze manier het afweersysteem af te leiden. In reactie op dit veranderde virus maakt het afweersysteem weer nieuwe neutraliserende antistoffen, maar
ook hieraan kan het virus weer ontsnappen door kleine veranderingen in het envelop-eiwit. Zo blijft het afweersysteem steeds achter de feiten aanlopen.

Antistoffen die zich niet richten op de variabele maar juist op de geconserveerde delen worden maar door een beperkt aantal hiv-geïnfecteerde patiënten gemaakt. Deze breed neutraliserende antistoffen kunnen virussen die erg van elkaar verschillen en zelfs virussen van andere subtypen herkennen. Deze antistoffen zijn erg interessant voor vaccinontwikkeling, maar helaas is er nog geen manier gevonden om deze breed neutraliserende antistoffen op te wekken door middel van een vaccin.

**DIT PROEFSCHRIFT**

In dit proefschrift wordt de prevalentie, ontwikkeling en eigenschappen van het breed neutraliserend vermogen in serum van hiv-geïnfecteerde patiënten onderzocht. Daarnaast hebben we bestudeerd via welk mechanisme het virus kan ontsnappen aan neutraliserende antistoffen in het lichaam.

In **hoofdstuk 2** hebben we het neutraliserende vermogen in sera van 35 patiënten uit de Amsterdam Cohort Studies bekeken. Twee jaar na de infectie met hiv-1 subtype B heeft het serum van ongeveer 20% van de patiënten het vermogen om verscheidende hiv-1 virussen van verschillende subtypen te neutraliseren en iets meer dan 60% van de patiënten heeft serum met het vermogen om meerdere hiv-1 virussen van subtype B te neutraliseren. Deze subtype-specifieke neutraliserende antistoffen en hun relevantie voor een mogelijk hiv-vaccin, worden in meer detail besproken in **hoofdstuk 5**. Het percentage patiënten met breed neutraliserend vermogen in serum is vier jaar na infectie toegenomen tot ongeveer 30% (**hoofdstuk 3**). Ook zien we een verband tussen het gehalte aan antistoffen in het bloed en het vermogen om verschillende virussen te kunnen neutraliseren. Het serum van nog eens 82 hiv-geïnfecteerde patiënten uit de Amsterdam Cohort Studies is onderzocht op breed neutraliserend vermogen (**hoofdstuk 4**) en ook in deze grote studie blijkt dat ongeveer 30% van de hiv-geïnfecteerde patiënten het vermogen ontwikkelt om verscheidende hiv-1 virussen van verschillende subtypen te neutraliseren en dat het twee tot drie jaar duurt voordat dit breed neutraliserend vermogen is ontwikkeld. Verder zagen we een positief verband tussen de hoeveelheid virus in bloed en de ontwikkeling van breed neutraliserend vermogen in serum. Ook een lager aantal CD4+ T cellen, zelfs al voordat de hiv-infectie zich voordoet, is sterk gecorreleerd met het breed neutraliserend vermogen in serum van hiv-geïnfecteerde patiënten. Dit kan er op duiden dat het afweersysteem een bepaalde hoeveelheid virus moet zien om breed neutraliserende antistoffen te kunnen maken en dat dit onder andere te maken heeft met een gevoelige balans tussen de verschillende cellen van het afweersysteem. Uit deze studie blijkt helaas ook dat het hebben van breed neutraliserend vermogen in serum op de lange termijn geen invloed heeft op het ziekteverloop.

Het onvermogen van neutraliserende antistoffen om het ziekteverloop te remmen, komt waarschijnlijk doordat hiv-1 relatief gemakkelijk kan ontsnappen aan neutraliserende
antistoffen. In **hoofdstuk 7** zien we dat dit zelfs gebeurt in patiënten met breed neutraliserend vermogen in serum. Ondanks het feit dat breed neutraliserende antistoffen gericht zijn op de geconserveerde delen van het envelop-eiwit, zien we dat de ontsnapping van hiv-1 aan breed neutraliserende antistoffen gepaard gaat met veranderingen in de variabele delen van het envelop-eiwit. De variabele delen worden langer en er zijn meer suikers gebonden aan deze delen, waardoor de gebieden die de antistoffen herkennen, afgeschermd worden. Om in meer detail naar de evolutie van het hiv-1 virus te kijken binnen een patiënt hebben we op verschillende momenten na infectie virussen uit cellen en serum onderzocht (**hoofdstuk 6**). In sommige patiënten zagen we dat sommige virussen een iets ander envelop-eiwit hadden dan het overgrote deel van de virussen in die patiënt. **Hoofdstuk 10** laat zien dat dit hoogstwaarschijnlijk wordt veroorzaakt door de druk die de aanwezige antistoffen in de patiënt uitoefenen op het virus. Doordat er continue ‘foutjes’ ontstaan tijdens het maken van nieuwe hiv-1 virussen komen er veel verschillende virussen tegelijkertijd voor in één patiënt. De antistoffen die aanwezig zijn in de patiënt ruimen sommige van deze virussen op, maar de virussen die niet worden herkend door de antistoffen kunnen weer nieuwe cellen infecteren en zo verder evolueren.

We zien bovendien dat hiv-1 virussen die eigenlijk niet zeer sterk onder druk staan van de antistoffen toch veranderingen krijgen in het envelop-eiwit. Deze veranderingen kunnen er eveneens voor zorgen dat de hiv-1 virussen niet meer worden herkend door breed neutraliserende antistoffen (**hoofdstuk 8 en 9**). Hieruit blijkt dat ook andere factoren ervoor kunnen zorgen dat het envelop-eiwit verandert en dat het virus daardoor (per ongeluk) ook beschermd is tegen antistoffen. In **hoofdstuk 11** wordt beschreven dat de bescherming tegen antistoffen voornamelijk wordt veroorzaakt door veranderingen in de variabele gebieden 1 en 2 (V1V2) van het envelop-eiwit. Deze gebieden vormen samen een grote lus die over de CD4-receptorbindingsplaats ligt. Als deze lus groter wordt en/of meer suikers bevat is het virus beter beschermd tegen antistoffen, voornamelijk antistoffen gericht op de CD4-receptorbindingsplaats.

De resultaten beschreven in dit proefschrift laten zien dat het menselijk lichaam in staat is antistoffen te maken die hiv-1 virussen van verschillende subtypen kunnen herkennen. Helaas is hiv-1 in staat zich aan te passen aan zelfs deze breed neutraliserende antistoffen. Een mogelijk hiv-vaccin zou idealiter breed neutraliserende antistoffen moeten opwekken en hiermee moeten voorkomen dat het virus cellen kan infecteren en gaat vermenigvuldigen zodat het niet de mogelijkheid krijgt om aan deze antistoffen te ontsnappen.
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CURRICULUM VITAE

Marit van Gils was born in Gouda, The Netherlands on January 17, 1982. After graduating from secondary school (VWO, Dongemond College, Raamsdonksveer, The Netherlands) in 2000, she moved to Groningen to study Medical Pharmaceutical Sciences at the University of Groningen, The Netherlands. Her first traineeship was at the department of Pharmacokinetics and Drug Delivery under the supervision of Prof. dr. Geny Groothuis. For this traineeship she studied a multicellular in vitro model as an alternative to animal models. In June 2004 she obtained her Batchelor’s degree. For her Master’s degree she did a second traineeship at the department of Pharmacokinetics and Drug Delivery and a traineeship at AgResearch, Palmerston North, New Zealand under the supervision of Dr. Albert Koulman. There she worked on the symbiosis of endophyte-infected Ryegrass to unravel antibacterial peptides. After traveling around New Zealand and Australia for three months she finished her Master’s degree in August 2006. Subsequently she work as a technician at the department of Pharmacokinetics and Drug Delivery for three months until she started as a PhD student at the department of Clinical Viro-Immunology at Sanquin Research, Amsterdam, The Netherlands in February 2007. In December 2007 the department moved to the Academic Medical Center, Amsterdam, The Netherlands and was incorporated into the department of Experimental Immunology. The results of her research project ‘Characterization and optimization of HIV-specific humoral immunity’ are presented in this thesis book.
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