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Multiple Pathways of Escape from HIV Broadly Cross-Neutralizing V2-Dependent Antibodies

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Broadly cross-neutralizing (BCN) antibodies are likely to be critical for an effective HIV vaccine. However, the ontogeny of such antibodies and their relationship with autologous viral evolution is unclear. Here, we characterized viral evolution in CAP256, a subtype C-infected individual who developed potent BCN antibodies targeting positions R166 and K169 in the V2 region. CAP256 was superinfected at 3 months postinfection with a virus that was highly sensitive to BCN V2-dependent monoclonal antibodies. The autologous neutralizing response in CAP256 was directed at V1V2, reaching extremely high titers (>1:40,000) against the superinfecting virus at 42 weeks, just 11 weeks prior to the development of the BCN response targeting the same region. Recombination between the primary and superinfecting viruses, especially in V2 and gp41, resulted in two distinct lineages by 4 years postinfection. Although neutralization of some CAP256 clones by plasma from as much as 2 years earlier suggested incomplete viral escape, nonetheless titers against later clones were reduced at least 40-fold to less than 1:1,000. Escape mutations were identified in each lineage, either at R166 or at K169, suggesting that strain-specific and BCN antibodies targeted overlapping epitopes. Furthermore, the early dependence of CAP256 neutralizing antibodies on the N160 glycan decreased with the onset of neutralization breadth, indicating a change in specificity. These data suggest rapid maturation, within 11 weeks, of CAP256 strain-specific antibodies to acquire breadth, with implications for the vaccine elicitation of BCN V2-dependent antibodies. Overall these studies demonstrate that ongoing viral escape is possible, even from BCN antibodies.

Neutralizing antibodies (NAbs) develop in almost all HIV-1-infected individuals in the first months following infection (1–3). However, these early NAbs are extremely strain specific, neutralizing only the autologous viruses from that individual (1–3). This is because they target the variable regions of the viral envelope, including the V1V2 and C3V4 regions (4–7). Viral escape from these strain-specific NAbs occurs rapidly, thus circulating viruses are seldom neutralized by contemporaneous sera, though they are sensitive to subsequent waves of NAbs (1, 2, 4, 5, 7–9). This process, which is ongoing for many years, results in envelope diversification and the generation of viral quasispecies that often show differences in neutralization sensitivity.

The development of broadly cross-neutralizing (BCN) antibodies, those with the capacity to neutralize heterologous viruses across many genetic subtypes, occurs only in about a quarter of HIV-1-infected people (10–15). Recently, there has been intense interest in mapping the targets of BCN antibodies in polyclonal sera and in isolating and characterizing BCN monoclonal antibodies (MAbs) from infected subjects. These studies have shown that the majority of the BCN activity is due to antibodies that target four sites on the HIV-1 envelope. These include the CD4 binding site (defined by the MAbs IgG1b12, VRC01, HJ16, and CH31), the gp41 membrane-proximal external region (MAbs 4E10, 2F5, and 10e8), a peptidoglycan epitope at the base of the V3 loop (MAbs 2G12, PG121, PGT128, and PGT135), and a peptidoglycan epitope in the V2 region (MAbs PG9, PG16, PGT141-145, and CH01-04) (1, 14–20). Since the epitopes defined by these MAbs represent vulnerable sites on the HIV-1 envelope, understanding the ontogeny of these types of antibody specificities could help to develop a preventative HIV vaccine that emulates this process.

To date, the development of BCN antibodies has been shown to be associated with the duration of infection, high viral load, low $CD4^+$ T cell counts, and viral diversity and evolution (10–13, 21, 22), the latter suggesting that viral factors play a key role in this process. It is not known whether BCN antibodies arise by affinity maturation of earlier strain-specific NAbs or whether neutralization breadth is a consequence of *de novo* specificities, which through chance target more conserved epitopes. The fact that BCN antibodies normally take 2 to 3 years to appear (11) and the high levels of somatic hypermutation displayed by many BCN MAbs suggests a requirement for antibody maturation rather than simply a stochastic event. While the V1V2 and C3 regions are targets of both strain-specific early antibodies and later BCN antibodies (4–7, 11, 14, 15, 23, 24), the relationship between these antibodies and their impact on autologous viral evolution has not been well characterized. Data on viral escape from antibodies tar-

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geting conserved epitopes is limited, as many individuals who develop BCN antibodies were identified in cross-sectional cohorts of chronically infected individuals. In a recent study of autologous viral populations in the subject from whom the VRC01 MAb was isolated, efficient and ongoing viral escape, with viruses resistant to contemporaneous neutralization, was described in parallel with ongoing evolution of the BCN CD4 binding site antibody response (25). The possibility of viral escape, even from BCN antibodies targeting highly conserved epitopes, is consistent with the fact that the development of such antibodies does not confer a clinical benefit to those individuals (10, 11). This further suggests that viral escape from such responses does not incur significant fitness costs, as has been suggested elsewhere (26). Nonetheless, understanding viral evolution and defining the pathways to viral escape in individuals who develop BCN antibodies may provide insights into how such antibodies are elicited.

The V1V2 region was known to contain conserved elements that are recognized by BCN NAbs. This was confirmed by the recent structural analysis of a scaffolded subtype C V1V2 bound to the BCN MAb PG9 (27). This structure showed that V1V2 forms a four-stranded β-sheet domain, in which sequence variation and glycosylation are largely limited to loops that connect the strands, although alternative conformations of V1V2 may exist (28). Interest in the V2 region intensified following the case control analysis of the RV144 Thai vaccine trial that exhibited moderate (31%) efficacy, which identified V2 binding antibodies as a correlate of protection (29). Sieve analysis of break-through infections suggested immune pressure at residue 169 in the C beta strand of the V2 region (30), and MAbs isolated from RV144 vaccinees showed dependence on K169 (28). This finding, along with the previously defined dependence of the BCN MAbs PG9/PG16 (15, 31) and polyclonal BCN plasma (31) on this residue, suggests a need to better understand antibodies targeting this region.

We have previously described CAP256, a subtype C superinfected individual who developed potent BCN antibodies targeting a quaternary neutralizing epitope involving the V2 region (31), similar to the PG9 and PG16 MAbs (15). Fine mapping showed that the CAP256 BCN antibody was dependent on residues F159, N160, L165, R166, D167, K169, and K171 (forming the FN/LRD-K-K motif). Interestingly, the fine specificity of the evolving antibody response changed over time, including dependence on the glycan at position 160 that is crucial for recognition by PG9 and PG16 (31). Here, we have examined viral populations in CAP256 and characterized different pathways of neutralization escape from autologous antibodies. We show that the early strain-specific response, like the BCN response, targets the V1V2 region of both the primary infecting (PI) virus and the superinfecting (SU) virus. However, the response was largely directed at the superinfecting virus with extremely high titers, reminiscent of CAP256 heterologous neutralization. We further showed that in the context of extensive recombination, neutralization escape occurred via amino acid substitutions at residues 166 and 169 in the FN/LRD-K-K motif. These data provide insights into how HIV evades neutralizing antibodies that target the V2 region.

MATERIALS AND METHODS

CAPRISA participant CAP256. CAP256 was enrolled into the CAPRISA Acute Infection study (32) that was established in 2004 in KwaZulu-Natal, South Africa. This study was reviewed and approved by the research ethics committees of the University of KwaZulu-Natal (E013/04), the University of Cape Town (025/2004), and the University of the Witwatersrand (MM040202). CAP256 provided written informed consent for study participation. The kinetics and specificity of BCN antibodies in CAP256 has been described in detail elsewhere (11, 31).

SGA and sequencing of CAP256 envelope genes. HIV-1 RNA was purified from plasma using the Qiagen Viral RNA kit and reverse transcribed to cDNA using Superscript III Reverse Transcriptase (Invitrogen, CA). The *env* genes were amplified from single-genome templates as described previously (33). Amplicons were directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3100 automated genetic analyzer. The full-length *env* sequences were assembled and edited using Sequencher v.4.0 software (Genecodes, Ann Arbor, MI). For strain-specific single-genome amplification (SGA) of the CAP256 superinfecting virus, reverse transcription was performed using a primer specific for the superinfecting variant (256spR; 5′-CTCCCTCTGCTGTTGG CTGCGCTCGCGC-3′; HXB2 positions 8856 to 8884; Nef). SGA was performed as described above, using the strain-specific primer as the antisense primer in both rounds of amplification. Multiple-sequence alignments were performed using Clustal X (version 1.83) and edited with BioEdit (version 5.0.9). Sequence alignments were visualized using Highlighter for Amino Acid Sequences v1.1.0 (beta) (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/HIGHLIGHT_POSTSCRIPT/highlighter.html). Recombination was assessed using the RIP tool (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). Pairwise DNA distances and neighbor-joining trees were computed using MEGA 4 (34). Three genotypic coreceptor prediction algorithms, namely, C-PSSM (35), geno2pheno (36), and the 11/25 rule (37), were used to predict the coreceptor use of each envelope sequence.

Cloning CAP256 envelopes and production of pseudoviruses. Selected amplicons were cloned into the expression vector pCDNA 3.1 (directional) (Invitrogen) by reamplification of SGA first-round products using Phusion enzyme (Finn Enzymes) with the EnvM primer (38) and a directional primer, EnvAdir (4). Cloned *env* genes were sequenced as described above. The full-length gp160 sequences were assembled and edited using Sequencher v.4.0 software (Genecodes, Ann Arbor, MI). Cloned envelopes were shown to match the amino acid sequences of SGA-derived sequences exactly.

Cell lines. The JC53bl-13 cell line, engineered by J. Kappes and X. Wu, was obtained from the NIH AIDS Research and Reference Reagent Program. 293T cells were obtained from George Shaw (University of Alabama, Birmingham, AL). Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL Life Technologies) containing 10% heat-inactivated fetal bovine serum and 50 μg/ml gentamicin (Sigma). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA.

TZM-bl neutralization assays. Env-pseudotyped viruses were obtained by cotransfecting the Env plasmid with pSG3ΔEnv (3) using Fugene transfection reagent (Roche) as previously described (1). Neutralization was measured as described previously (1) by a reduction in luciferase gene expression after single-round infection of JC53bl-13 cells with Env-pseudotyped viruses. Titers were calculated as the reciprocal plasma dilution causing a 50% reduction of relative light units (ID_{50}).

Generation of chimeras and mutant envelopes. Chimeric V1V2 envelopes were created using an overlapping PCR strategy with the inserts and flanking regions amplified in separate reactions. After linkage, the 3-kb chimeric PCR fragments, generated using primers EnvAdir and EnvM (38), were cloned into pCDNA 3.1 (directional) (Invitrogen) and screened for function as previously described (39). Chimerism was confirmed by sequence analysis. Site-directed mutagenesis was performed using the Stratagene QuikChange II kit (Stratagene) as recommended by the manufacturer.

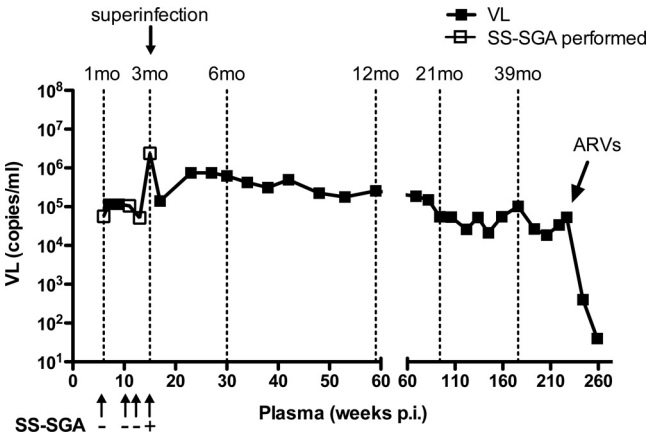


FIG 1 Schematic indicating the CAP256 viral load (VL; in RNA copies/ml) from enrollment to 4 years p.i. Dotted lines indicate the time points at which single-genome amplification (SGA) and sequencing was performed. Open squares indicate the samples used for strain-specific SGA to determine the timing of the superinfection. Arrows indicate the time point at which clones were amplified. ARVs, start of antiretroviral treatment.

RESULTS

Superinfection of CAP256 by a subtype C virus shortly after primary infection. CAP256 is a subtype C-infected individual in the CAPRISA 002 cohort who developed potent BCN antibodies that preferentially neutralized subtype C viruses (11, 31). Mapping of the BCN antibodies at 3 years of infection showed that they targeted residues in the V2 region within the context of the envelope trimer, similar to the PG9/16 MAbs. Despite the development of BCN antibodies, CAP256 required antiretroviral treatment at 4 years of infection after developing tuberculosis (Fig. 1).

In order to examine viral escape from the BCN response in CAP256, SGA-derived envelope sequences were generated from plasma at 1 month (enrollment into the study) and at 6, 12, 21, and 39 months postinfection (p.i.) (Fig. 1; sequence alignment of the complete V1V2 is shown in Fig. S1 in the supplemental material). Sequence analysis showed unusually high genetic variability (up to 10.8% in *gp160*) by 6 months of infection that was inconsistent with the short duration of infection. This, in conjunction with the observation that the viral load of CAP256 transiently increased nearly 50-fold from 51,600 copies/ml at 13 weeks p.i. to 2,390,000 copies/ml at 15 weeks before declining again to 141,000 copies/ml by 17 weeks (Fig. 1), suggested the possibility of superinfection.

To identify the potential superinfecting virus, a strain-specific primer was designed based on the sequences of later presumed recombinant viruses. Strain-specific single-genome PCR amplification (SS-SGA) was performed using plasma from 6, 11, 13, and 15 weeks p.i., corresponding to the earliest available sample and those prior to and at the peak of the viral load spike at 15 weeks p.i. (Fig. 1). While no amplification was evident in the samples from 6, 11, and 13 weeks p.i., a phylogenetically distinct envelope was successfully amplified at 15 weeks p.i., suggesting that superinfection occurred between 13 and 15 weeks after the initial infection. Consistent with this, the 6 amplicons sequenced from 15 weeks were highly homogenous, suggesting that they had been recently transmitted. Furthermore, there was no evidence of recombination with the initial virus at this time point using the Recombination Identification Program (RIP) of the Los Alamos Sequence Database, with a window size of 100 bp and a background alignment, including sequences from the initial virus (data not shown).

The superinfecting virus is highly sensitive to early CAP256 NAbs. In order to measure viral escape from BCN NAbs and earlier strain-specific responses, selected amplicons were cloned from each time point and used to generate functional pseudoviruses for use in the TZMbl neutralization assay. Clone 256.1mo.C7 matched the consensus sequence from the enrollment time point and was assumed to reflect the transmitted/founder virus of the primary infecting (PI) virus. Similarly, clone 256.3mo.9C matched the consensus of the 3-month strain-specific SGA and was assumed to represent the transmitted/founder superinfecting (SU) virus. Pseudotyped viruses were assessed for neutralization sensitivity against longitudinal plasma collected over 21 time points, at least every 3 months from enrollment to 4 years p.i. The neutralizing antibody response against each envelope clone, including the PI and SU viruses, is shown in Fig. 2A, with the colored arrows indicating the time point from which clones in the matching color were amplified.

Neutralizing antibodies were first detected at 23 weeks postinfection, targeting both the PI and SU viruses with similarly low titers (ID_{50} of approximately 1:300). Titers against the PI virus remained at approximately this level for the next 4 years. In contrast, the NAb response against the SU virus became considerably more potent, reaching extremely high titers of >1:45,000 by 69 weeks of infection. This sudden increase suggested the development of a novel neutralizing specificity directed predominantly at the superinfecting virus.

Titers against viruses cloned from later time points were also measured using longitudinal plasma. None of the later clones exhibited sensitivity matching that of the SU virus, with peak titers against the later clones generally being less than 1:4,000. The timing of the response against the 6-month clones (pink) matched that of the high-titer response targeting the SU virus, although titers were substantially lower. Neutralization curves for the 12- and 21-month viruses were shifted to the right, with little or no contemporaneous neutralization, consistent with ongoing neutralization escape. However, interestingly, many of the 39-month clones showed no further shift rightwards compared to the 21-month clones and exhibited increased neutralization by contemporaneous plasma. Indeed, some 39-month clones were sensitive to neutralization (albeit at low titers) by plasma from as much as 100 weeks earlier (Fig. 2A). This finding of contemporaneous neutralization, and even neutralization of later viruses by plasma from much earlier time points, was suggestive of incomplete viral escape.

CAP256 NAbs target the V1V2 region of both infecting viruses. Given that we had previously shown that the BCN response in CAP256 was directed at the V1V2 region (31), we asked whether the overall autologous NAb response to CAP256 viruses was also directed at this region. We therefore constructed chimeric viruses, whereby the V1V2 regions of the PI and SU viruses were transferred into a neutralization-resistant heterologous virus, CAP84 (4). Chimeric viruses were tested against autologous plasma for neutralization sensitivity and compared to the parental viruses (Fig. 2B).

The chimera containing the V1V2 region from the PI virus showed low-level neutralization sensitivity but only from 42 weeks p.i. onwards, with the neutralization profile thereafter matching that of the parental virus (Fig. 2B, compare gray curves). As the autologous NAb response against the PI virus was detect-

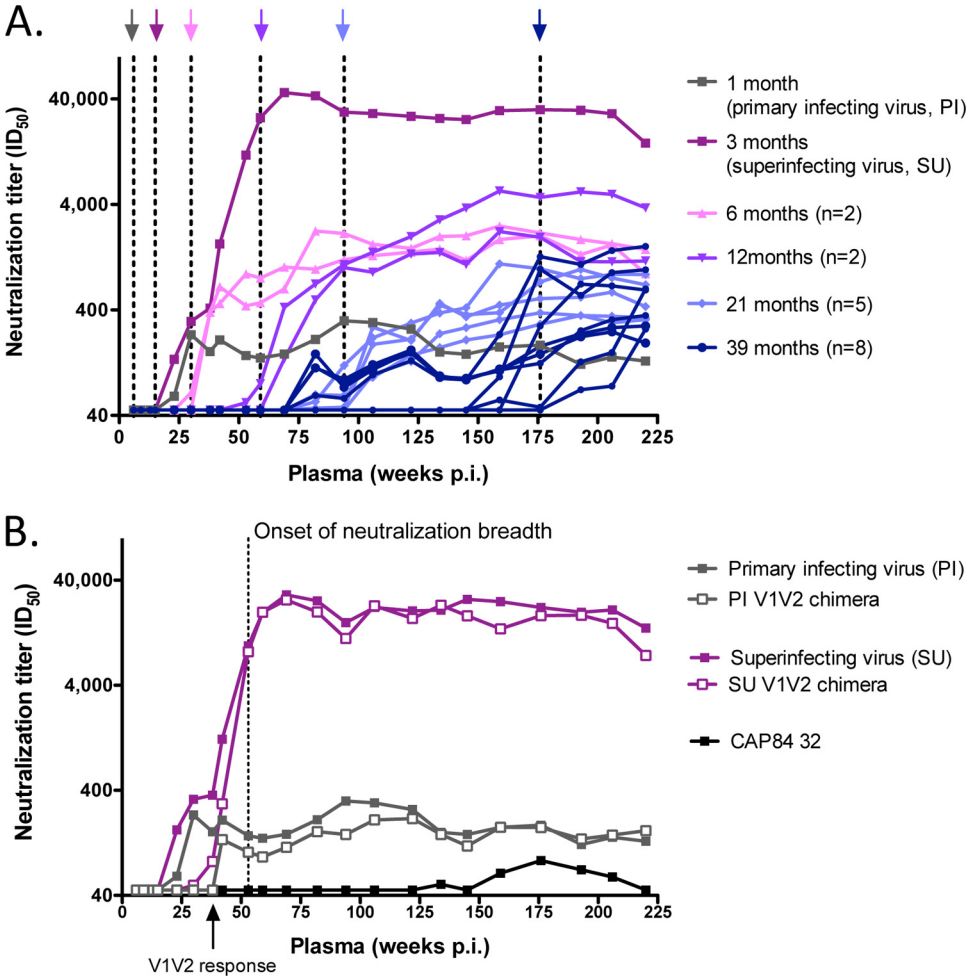
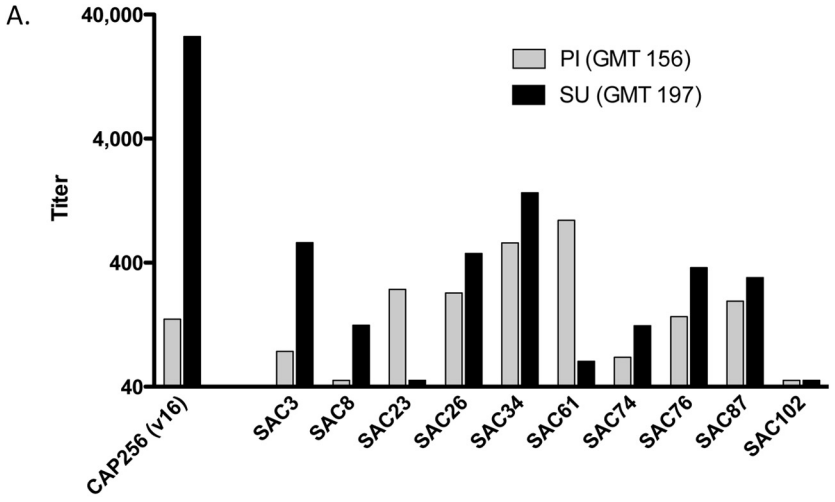


FIG 2 (A) Neutralization escape in CAP256. Neutralization sensitivity of multiple autologous CAP256 Env-pseudotyped viruses derived from envelope clones at 1, 3, 6, 12, 21, and 39 months postinfection. Each clone was tested against longitudinal autologous plasma. Colored arrows indicate the time point at which clones were amplified. Neutralization titers (ID_{50}) are plotted on a logarithmic scale. (B) The autologous neutralizing response is largely directed against V1V2. Chimeric constructs containing only V1V2 on both the initial infecting and superinfecting viruses, placed in a neutralization-resistant backbone, were used to generate Env-pseudotyped viruses, which were tested against longitudinal CAP256 plasma. The onset of the V1V2 response and of the BCN antibodies (31) are shown with dotted lines.

able earlier, at 23 weeks p.i., this suggested an initial neutralizing response of unknown specificity, which was followed by a second low-titer NAb response mediated by anti-V1V2 antibodies. Similarly, the SU V1V2 chimera became sensitive to neutralization at 38 weeks p.i., thereafter showing high-level sensitivity almost exactly matching that of the parental virus (Fig. 2B, compare purple curves). As with the PI virus, the SU virus was also weakly neutralized initially by an unknown specificity. This was followed by an extremely high-titer V1V2 response. Therefore, like the BCN response in CAP256, the overall autologous neutralizing response from 38 weeks to 4 years postinfection was mediated largely by anti-V1V2 antibodies.

The superinfecting virus is highly sensitive to BCN anti-V2 NAbs. The observation that the PI and SU viruses were sensitive to CAP256 plasma but that the SU virus was 1,000 times more sensitive led us to question the overall neutralization phenotype of these two distinct primary viruses. Both the PI and SU virus envelope clones were tested against 10 HIV^+ plasma samples obtained from chronically infected individuals through the South African National Blood Services (40). Figure 3A shows that while some plasma samples preferentially neutralized one virus over the other (e.g., SAC3 neutralized SU virus better than the PI virus, and SAC61 preferentially neutralized PI over SU virus), overall there was no significant difference in their neutralization sensitivity (geometric mean titer of 197 for the SU virus and 156 for the PI virus), suggesting that both exhibited a tier-2 neutralization phenotype (41). These viruses were also tested against monoclonal antibodies targeting CD4bs (b12 and VRC01), MPER (4E10), a glycan-dependent epitope in C3 (PGT128), and the V2 loop (PG9, PG16, and PGT145) (Fig. 3B). The PI virus, but not the SU virus, was sensitive to b12, although both viruses were neutralized by the more potent VRC01 and were also equivalently sensitive to 4E10 and PGT128. However, in contrast, the SU virus was nearly 1,000 times more sensitive to PG9 and nearly 10,000 times more sensitive to PG16, both of which target the conserved elements in the V2 loop. PGT145 showed a similar, though less striking, trend, with the SU virus being 27 times more sensitive than the PI virus. Therefore, the much higher anti-V2 titers in CAP256 plasma



B.

specificity	CD4bs		MPER	N332	anti-V2, N160		
mAb (ug/ml)	b12	VRC01	4E10	PGT128	PG9	PG16	PGT145
PI	2.1344	0.1166	12.45	0.0882	44.8	>50	33.37
SU	>50	0.6811	3.59	0.0447	0.0453	0.0055	1.25

FIG 3 Comparison of the overall neutralization sensitivity of the primary infecting virus and the superinfecting virus against polyclonal subtype C plasma (A) and broadly neutralizing monoclonal antibodies (B). The geometric mean titer (GMT) of each virus is indicated in parentheses.

against the SU virus were determined by the presence and/or accessibility of the PG9/16 epitope on the SU virus compared to the PI virus, rather than more universal determinants of sensitivity, such as those exhibited by tier-1 viruses (41).

Recombination in V1V2 and gp41 drives rapid evolution of CAP256 sequences. We examined the evolution of CAP256 viral sequences over time in the context of the known BCN antibody response targeting the V2 region. Figure 4A shows an amino acid highlighter plot illustrating the relationship between the PI virus (shown with gray shading), the superinfecting strain, and later viruses. By 6 months, the sampled viral population was comprised entirely of recombinant viruses, with most of the envelope (except the V1V2 region and part of the carboxyl terminus of the gp41) derived from the superinfecting virus. By 12 months, multiple different recombinant populations existed. Approximately two-thirds of the 12-month viruses contained V1 and part or all of V2 from the PI virus. The remaining 12-month sequences contained the V1V2 region from the superinfecting virus, with the gradual accumulation of amino acid mutations within the V1V2 regions. In addition, many 12-month viruses also contained the C5 region of gp120, the gp120-gp41 cleavage site, the gp41 fusion peptide, and other isolated parts of gp41 from the immunodominant region, through the carboxy-terminal heptad repeat region and membrane proximal external region but excluding the amino-terminal heptad repeat region. By 21 and 39 months postinfection, all but 1 sequence contained the bulk of gp41 from the PI virus, suggesting a benefit to the recombinant virus in maintaining this segment. However, interestingly, both viruses with the entire V1V2 region derived from the PI or SU virus persisted throughout the course of infection. All viral clones were predicted by three coreceptor prediction tools to use the CCR5 coreceptor throughout the course of infection.

Evolution within the FN/LRD-K-K motif in V2, the target of CAP256 BCN antibodies. We have previously shown that the CAP256 BCN specificity depends on residues in the V2 region, namely, F159, N160, L165, R166, D167, K169, and K171 (forming the FN/LRD-K-K motif) (31). As the overall autologous response, like the BCN specificity, was largely directed at V2, we assessed whether residues that formed the BCN epitope were mutated in later autologous viruses. Figure 4B shows a detailed amino acid highlighter analysis of the CAP256 V1V2 sequences, highlighting the location of the FN/LRD-K-K motif. This motif was present in the SU but was incomplete in the PI virus. By 6 months, all sequences had changes within the FN/LRD-K-K motif, which were introduced by recombination with the PI virus. However, from 12 months onwards, mutations in V2 and specifically in the FN/LRD-K-K motif occurred through recombination with the PI virus and/or through point mutations (absent from both the primary and superinfecting viruses).

By 39 months postinfection, the CAP256 viral population had formed two distinct clusters (Fig. 5). These two lineages could be clearly differentiated from one another both on the basis of neutralization sensitivity and by sequence changes within the FN/LRD-K-K motif. Cluster 1 clones (39mo.C2, 39mo.F10, 39mo.E1, and 39mo.H1) (Fig. 6A) were sensitive to neutralization at low titers by plasma from 2 years earlier. In contrast, cluster 2 clones (39mo.F1, 39mo.F11, 39mo.F10, and 39mo.F4) (Fig. 6B) exhibited a neutralization profile more consistent with complete neutralization escape, although two of these clones (39mo.F1 and 39mo.F11) also exhibited relatively high titers (ID_{50} of 282 and 973, respectively) when tested against contemporaneous plasma. Of note, clones 39mo.4 and 39mo.10 of cluster 2 were completely resistant to neutralization by contemporaneous plasma, emphasizing the fact that complete escape from the CAP256 BCN NAbs was possible. They did, however, show susceptibility to later plasma antibodies.

Viruses from cluster 1, epitomized by 39mo.C2, contained 6 mutations in the FN/LRD-K-K motif compared to the superin-

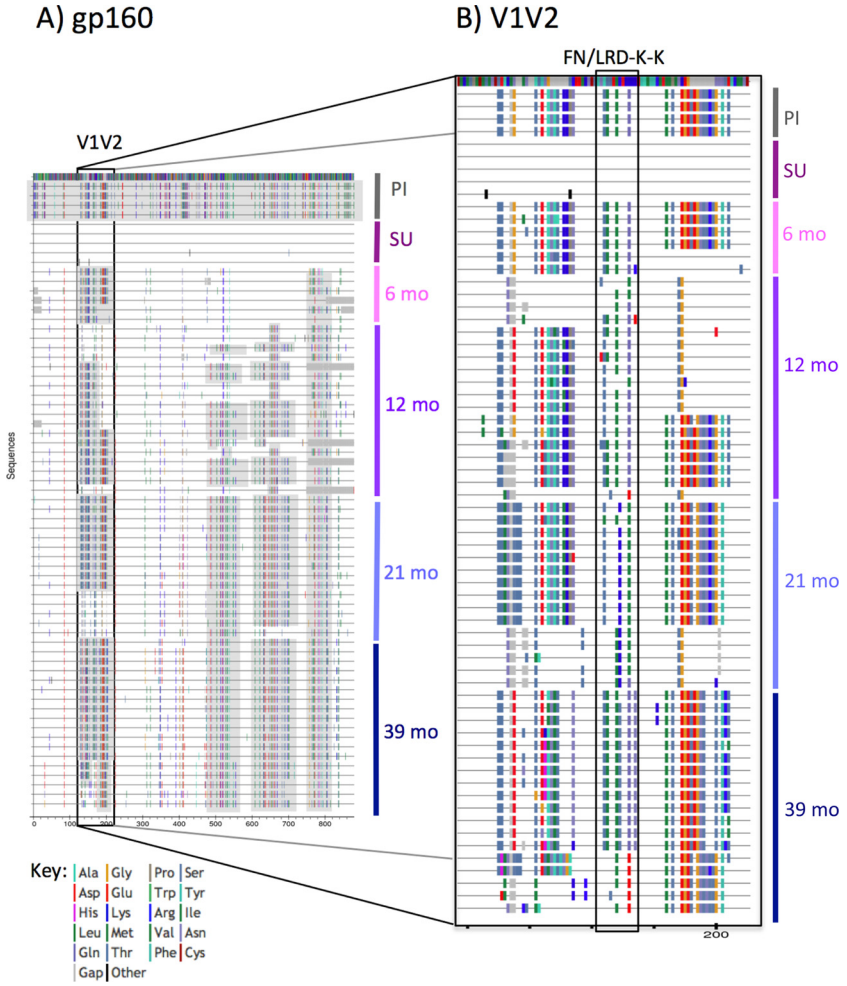


FIG 4 Amino acid highlighter plot comparing the initial infecting virus, the superinfecting virus, and viruses isolated from subsequent time points for the entire envelope gene (A) and specifically the V2 region, including the FN/LRD-K-K motif (B). Gray shading indicates regions matching the initial infecting virus and highlights recombination, which occurs predominantly in V1V2 and in gp41.

fecting virus (Fig. 6A). Four of these mutations (A161T, T162I, L165V, and K169Q) were present in the PI virus with identical codon usage, suggesting they were introduced through viral recombination. The observation that the PI virus was relatively resistant to neutralization (Fig. 2A) suggested that recombination provided a mechanism for neutralization escape. However, in addition to these 4 changes, cluster 1 sequences also contained an R166S and K171N mutation within this region that were not present in the PI virus and therefore presumably arose by substitution after recombination had occurred. In contrast, the viruses in cluster 2 had V2 regions that generally matched the superinfecting virus, with only two substitutions in the FN/LRD-K-K motif, namely, L165V (also present in the PI virus) (or T163S) in conjunction with K169E that arose by substitution.

Changes within the FN/LRD-K-K motif mediate viral escape from autologous NAb responses. We selected one clone from each lineage at 39 months, 256.39mo.C2 (cluster 1) and 256.39mo.F1 (cluster 2), to establish whether the changes observed in the region containing the FN/LRD-K-K region mediated neutralization escape. Mutations observed in the region spanning positions 159 to 171 (encompassing the FN/LRD-K-K motif) in either clone were introduced singly or in combination into the highly sensitive SU virus. These were used to generate pseudoviruses and tested for sensitivity to longitudinal autologous plasma to determine if they rendered the SU virus less sensitive to neutralization.

In the cluster 1 clone, the T162I change (which removes the glycan at position 160 that is important for binding to PG9/PG16-like antibodies) and the K171N mutations, introduced singly, had moderate effects on neutralization sensitivity, with titers reduced 4-fold from >1:31,000 to approximately 1:7,000 at 39 months p.i. (Fig. 7A). However, of note, the kinetics of the neutralization curves of these 2 mutants differed. The K171 mutation had a moderate effect on titers at all time points, while the effect of the T162I mutation was more pronounced before the onset of breadth (with titers reduced 18-fold from 11,760 to 645). Thereafter and corresponding with the onset of breadth, the effect of this mutation was reduced, with neutralization titers increasing 10-fold to 1:7,000, suggesting that the initial anti-V2 NAb depended more heavily on the glycan at position 160, while the later BCN antibodies had reduced dependence on the N160 glycan.

The K169Q mutation had a slightly greater effect on neutral-

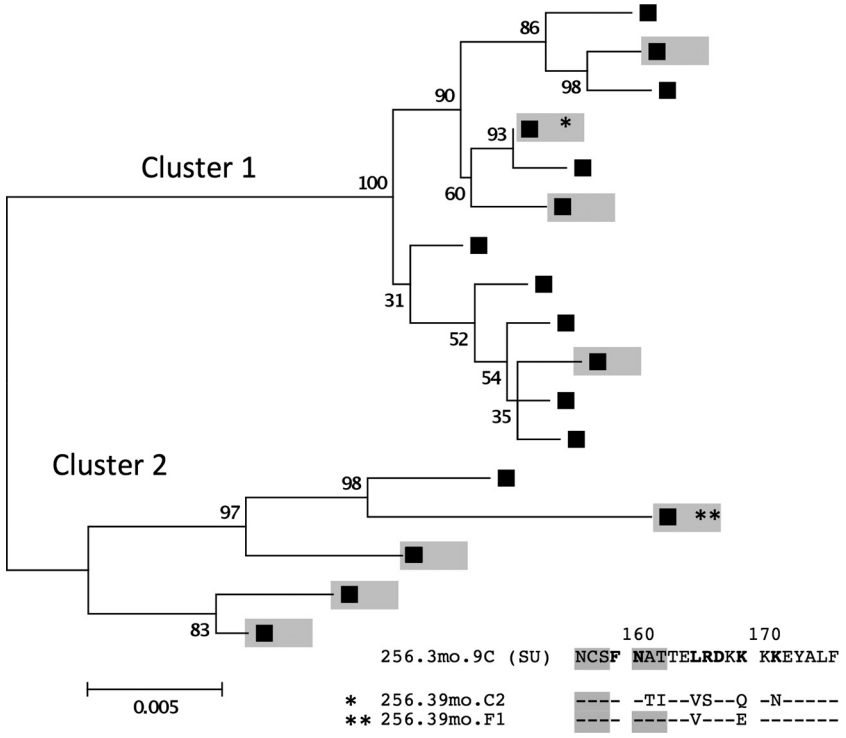


FIG 5 Phylogenetic tree of CAP256 showing the development by 39 months postinfection of distinct lineages, cluster 1 and cluster 2. Gray shading indicates sequences cloned for phenotypic assays. Inset are sequences of part of V2, including the FN/LRD-K-K motif for the SU virus, and a representative of cluster 1 (256.39mo.C2; indicated by an asterisk) and of cluster 2 (256.39mo.F1; double asterisks), highlighting potential escape mutations.

ization titers than either T162I or K171N, with a 7-fold decrease in titers at 39 months p.i. This effect waned slightly over 4 years of infection. In contrast, as previously reported for the BCN specificity (31), an R166S mutation consistently and profoundly reduced the neutralization titers 50-fold from 31,614 to 654 at the 39-month time point. The A161T mutation within the SU virus resulted in a virus unable to efficiently infect cells, therefore it could not be evaluated. We also tested constructs containing certain combinations of multiple mutations within the FN/LRD-K-K region. However, the introduction of K169Q and K171N along with the R166S mutation had no further effect on neutralization titers. Addition of the T162I mutation to the triple mutant resulted in a noninfectious virus that could not be tested. Overall, these data suggested that the most important escape mutation in cluster 1 viruses was the R166S mutation (Fig. 7A). Nonetheless, the residual neutralization of mutants suggests the presence of unidentified escape mutations elsewhere.

We similarly analyzed changes in the FN/LRD-K-K region in cluster 2 where only 2 mutations were observed, namely, L165V and K169E. As described above, these mutations were introduced

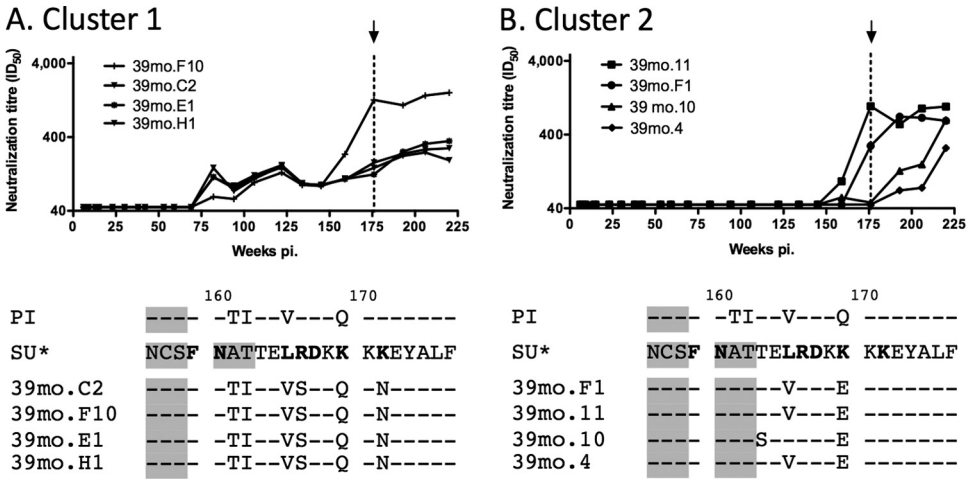


FIG 6 Comparison of the neutralization curves of 39-month viruses from cluster 1 (A) and cluster 2 (B). Partial V2 sequences for cloned viruses are shown below and are aligned to the superinfecting virus.

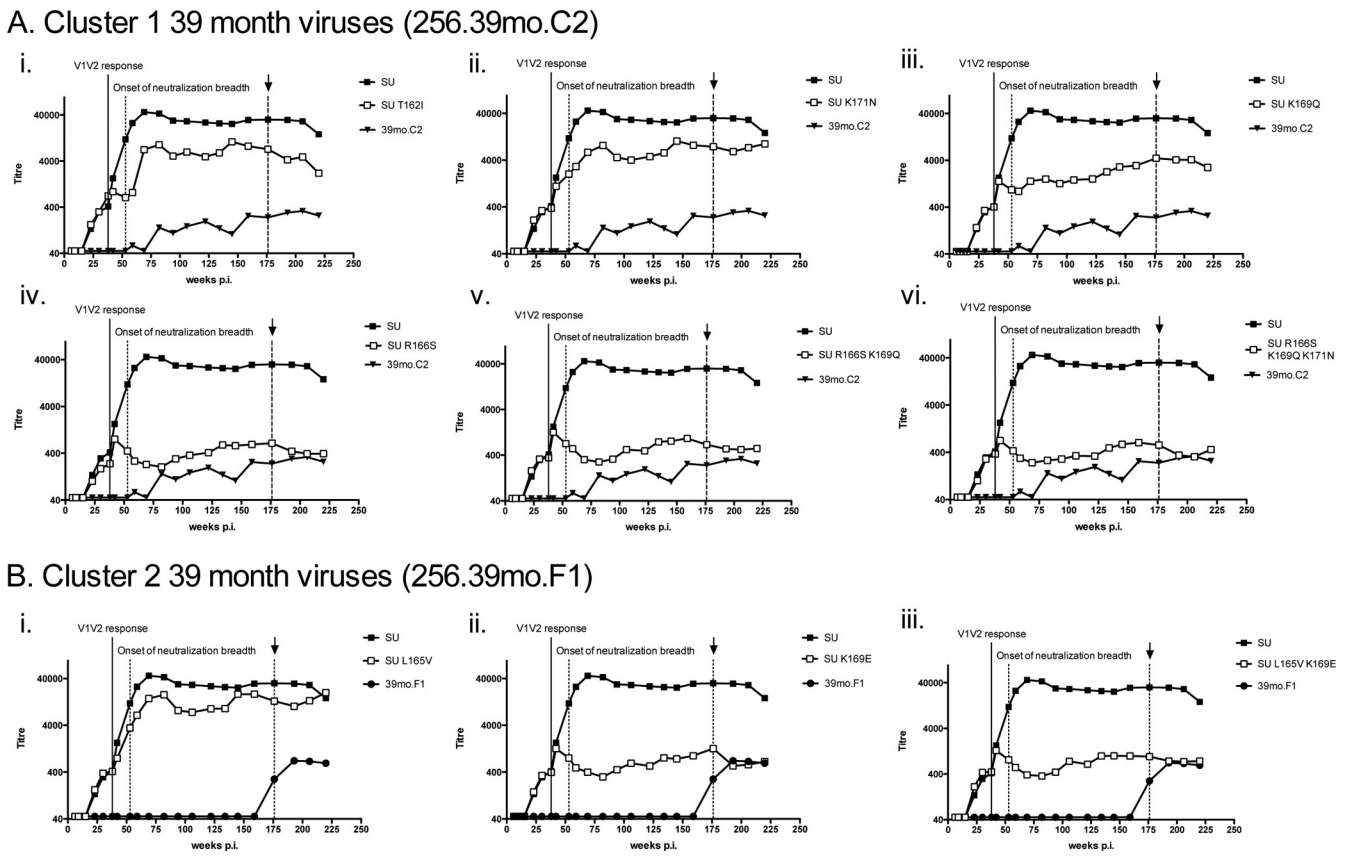


FIG 7 Mapping mutations that mediate neutralization escape in two representative 39-month clones, 256.39mo.C2 (cluster 1) (A) and 256.39mo.F1 (cluster 2) (B). Potential escape mutations were introduced by site-directed mutagenesis into the highly sensitive superinfecting virus and tested for neutralization sensitivity against longitudinal plasma samples from CAP256. Neutralization titers (ID_{50}) are plotted on a logarithmic scale.

singly and jointly into the sensitive SU virus. The L165V mutation had only a slight effect (2-fold) on neutralization titers (Fig. 7B). This substitution therefore did not appear to contribute significantly to viral escape in CAP256. In contrast, the effect of the K169E mutation was substantial, reducing titers to less than 1:1,000 at all time points. Combining the K169E mutation with the L165V mutation had no additional affect, suggesting that in cluster 2, viral escape from this specificity was driven largely by a single change at K169E, though as with cluster 1 viruses, residual neutralization sensitivity implicated residues yet to be identified elsewhere. Overall, CAP256 appeared to escape from the dominant V2-dependent NAb specificity through two distinct pathways, with mutations either at residue 166 (cluster 1) or 169 (cluster 2) within the V2 region.

The moderate resistance imparted by the K169Q change, which was present in many of the earlier sequences (see Fig. S1 in the supplemental material), suggested that recombination with the V2 region from the PI virus (containing the K169Q mutation) provided the SU virus with an initial pathway to viral escape, reducing sensitivity 10-fold. However, later clones escaped further through either an R166S or K169E mutation, further reducing sensitivity by 10-fold.

Mutations in the C4 region contribute to viral escape in some CAP256 viruses. To assess whether the unidentified remaining escape mutations lay within V1V2 or elsewhere, we constructed chimeras where the V1V2 region from the two 39-month viruses were transferred into a heterologous neutralization-resistant backbone, CAP84. Both chimeras, 39mo.C2.V1V2 (cluster 1) and 39mo.F1.V1V2 (cluster 2), were tested for neutralization sensitivity to longitudinal plasma and showed profound differences in neutralization sensitivity. The cluster 1 chimera was completely resistant to neutralization, even at later time points when the parental 39mo.C2 clone exhibited low-level sensitivity (Fig. 8A). This observation suggested (i) that the mutations aside from those identified in the FN/LRD-K-K motif but within the V1V2 region contributed to escape in this clone, and (ii) that the NAbs responsible for the low-level neutralization of cluster 1 viruses from 75 weeks onwards targeted a region other than V2.

In contrast, the cluster 2 chimera, despite the presence of K169E, exhibited substantial neutralization sensitivity, with titers exceeding 1:10,000, similar to that of the SU virus (Fig. 8B). This result was unexpected and suggested that additional residues outside V1V2 were required for escape. Inspection of the entire envelope sequence identified a change to alanine at position 437 in the C4 region of the envelope that was present only in cluster 2 sequences at 39 months. To define the location of residue 437 relative to V1V2, we fitted a crystal structure of clade C gp120 (PDB-3LQA) (42) into the cryo-electron microscopy (cryo-EM) structure of the envelope trimer in an unliganded state (EMD-5418) (43) using steepest ascent local optimization in UCSF Chimera 1.5.3 (44). The close proximity of residue 437 to the V1V2 region (Fig. 8C and D) supported the possibility that this residue

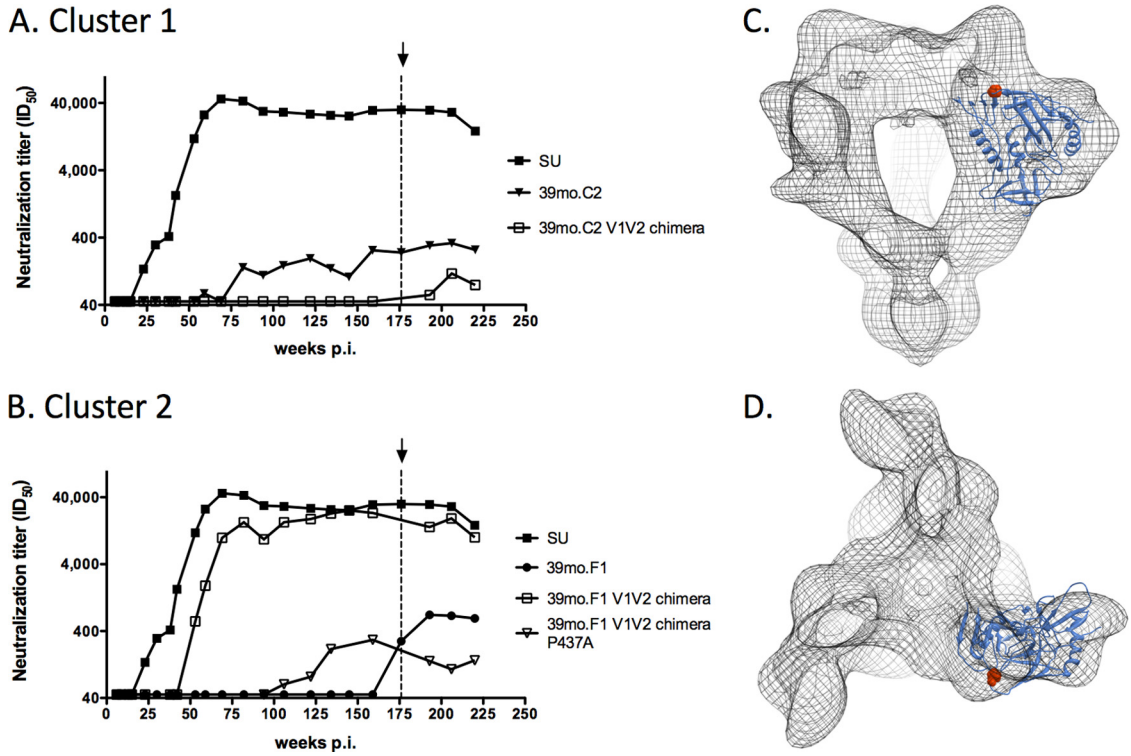


FIG 8 Use of chimeric viruses to determine whether neutralization escape is mediated solely by mutations in V1V2 for cluster 1 (A) and cluster 2 viruses (B). Neutralization titers (ID_{50}) are plotted on a logarithmic scale. The dotted line indicates the time point at which 39-month viruses were amplified (contemporaneous time point). Side (C) and top (D) views of the location of the V1V2 region and residue 437 (highlighted in red) within the envelope trimer, determined by fitting the crystal structure of clade C gp120 (in blue; PDB-3LQA) into the cryo-EM structure of the envelope trimer in an unliganded state (EMD-5418) (42–44).

affects neutralization escape. We therefore introduced a P437A substitution into the chimeric virus to create 39mo.F1.V1V2 P437A. This resulted in a significant drop in neutralization sensitivity, suggesting a role for the C4 region in mediating neutralization escape.

DISCUSSION

CAP256 was infected with an HIV-1 subtype C virus and developed extremely high-titer BCN antibodies recognizing a trimer-specific epitope in V2 of the envelope glycoprotein (15). In a previous study, we noted a low-titer autologous response to the primary infecting virus (1). However, here we used strain-specific primers to identify the envelope sequence of a second virus that superinfected CAP256 around 13 to 15 weeks after primary infection. We showed that like the BCN response, the overall NAb response to the superinfecting virus was directed almost exclusively at the V2 region, reaching titers similar to those observed against heterologous viruses. Viral evolution included extensive recombination between the primary and superinfecting viruses and resulted in neutralization escape through mutations in V2. However, low levels of contemporaneous neutralization were seen, indicating that viral escape from these BCN antibodies was incomplete. These data suggest that the BCN antibodies in CAP256 evolved from an earlier strain-specific NAb targeting V2.

We previously showed that antibodies with cross-neutralizing activity first appeared in the plasma of CAP256 at 53 weeks (31), only 11 weeks after strain-specific anti-V2 NAbs were detected. This rapid emergence of BCN antibodies following a strain-specific response targeting the same region suggested that these early V2-specific antibodies matured to acquire breadth. This was supported by our earlier observation that anti-V2 antibodies underwent subtle changes in specificity over time (31). In this study, the strain-specific autologous response was considerably more dependent on the glycan at position 160 than later BCN antibodies. We have previously shown differences in N160 neutralization-dependent heterologous neutralization by CAP256 plasma (e.g., neutralization of CAP45 was N160 dependent, whereas neutralization of ZM53 was not). Interestingly, the glycan at position 160 was not present on the primary infecting virus but was present on the superinfecting virus (21). Furthermore, at any given time point thereafter (even with relatively limited sampling using SGA), the circulating population included variants both with and without the predicted N-linked glycan at position 160. Thus, the continuous circulation of viruses heterogeneous for the position 160 glycan may have contributed to the development of BCN antibodies with variable dependence on this glycan. These data highlight the possibility that in CAP256, breadth was mediated by multiple antibodies, all of which were dependent on the residues at positions 166 and 169 but which differed in their glycan dependence (31). This, in conjunction with the fact that overall anti-V2 titers in CAP256 remained unchanged (which is inconsistent with the development of an entirely new high-titer specificity), supports the hypothesis that these BCN antibodies evolved from existing strain-specific NAbs rather than arising *de novo*. This would be encouraging from a vaccination perspective, as it suggests that

the immune system has the capacity to develop BCN V2-dependent antibodies in a relatively short time. Similar findings were recently reported from a simian-human immunodeficiency virus-infected macaque, where BCN antibodies developed within 9 months simultaneously with autologous NAb responses, both of which were mediated by N332 glycan-dependent antibodies (45). Both N160- and N332-dependent NAbs are commonly found among individuals who naturally develop BCN antibodies (11, 13, 23, 24). This finding therefore supports the suggestion by Walker et al. (45) that glycan-dependent antibodies are more rapidly elicited in natural infection and therefore may be more amenable to vaccine elicitation.

Two recent studies have shown that superinfection resulted in greater neutralizing breadth and potency (46, 47). In one of these studies, broad and potent NAb responses developed in 2 elite neutralizers very soon after infection with a second virus, suggesting that the development of breadth was accelerated through superinfection (46). Whether a similar situation provoked the exceptional breadth in CAP256 is unknown. It is possible that superinfection with a discordant virus results in the broadening of the NAb response to multiple epitopes presented on discordant viruses (48), a model supported by the fact that greatest breadth was associated with intersubtype superinfection (46, 47). It is also possible that superinfection focuses the NAb response onto conserved epitopes common to both infecting viruses. The latter scenario seems unlikely in the case of CAP256, however, as the secondary, high-titer NAb response which developed in response to the superinfecting virus did not result in any detectable increase in NAb titers toward the initial infecting virus. However, superinfection in CAP256 was associated with a massive increase in neutralizing titers directed to the superinfecting virus, similar to the response in a superinfected elite neutralizer, QA013, reported previously (49). This is in contrast to a recent study which showed no enhancement of autologous NAb responses in superinfected individuals (48). Overall, it is not clear if or how superinfection contributed to the exceptional breadth in CAP256.

What is clear is that superinfection in CAP256 resulted in extensive recombination and significant diversity in the envelope gene and probably facilitated neutralization escape. Interestingly, recombination was predominantly in two regions, with the highly sensitive superinfecting virus acquiring the V1V2 region and gp41 from the less sensitive primary virus. Given that the neutralizing response in CAP256 was almost entirely directed at V2, this suggested that recombination contributed to neutralization escape. Detailed characterization of the CAP256 viral populations identified two distinct viral lineages by 39 months of infection, with neutralization escape pathways differing between the two lineages. Cluster 1 viruses contained the V1V2 region of the primary virus in the superinfecting background, including a glutamine at residue 169, which was known to be crucial for the BCN epitope. However, although the presence of this glutamine reduced neutralization sensitivity somewhat, this mutation alone was not sufficient to mediate complete neutralization escape. A later R166S substitution substantially further reduced sensitivity. Thus, although recombination may have provided an initial, rapid pathway to reduce titers, this was not sufficient in the face of high-titer NAb responses to this region. In contrast, cluster 2 viruses escaped from the neutralizing response largely through a K169E substitution. Previous mapping data had shown that within the FN/LRD-K-K motif targeted by CAP256 BCN antibodies, the two positions most crucial for the formation of the epitope were R166 and K169. Mutations at either of these sites had a profound effect on BCN activity. In the ConC backbone, an R166A mutation resulted in a >100-fold reduction in neutralization, from a titer of 1:14,057 to 1:137. Similarly, a K169E mutation resulted in a 79-fold titer reduction to 1:179 (31). The observation that viral escape from autologous responses (by either of the two evolutionary pathways observed) occurred through mutations at positions 166 and 169 that form the basis of the BCN epitope (31) suggests that these responses targeted overlapping epitopes and further supports the notion that the strain-specific autologous response have evolved to acquire the capacity to neutralize heterologous viruses.

In addition to mutations at sites 166 and 169 in the V2 region, we also showed that a mutation at the highly conserved 437 residue in the C4 region contributed to viral escape. The presence of an alanine at position 437 is rare. Of 989 subtype C sequences from the Los Alamos sequence database, 967 (97.8%) have a P at position 437. The remaining sequences contain S ($n = 12$; 1.2%) or N ($n = 8$; 0.8%), with alanine present in only 2 sequences (0.2% of sequences). Furthermore, a linkage between E169 and mutations at position 437 is supported by the observation that of sequences with 169E, ~13% (7/54) have a residue other than P, compared to only 1.2% (8/653) in viruses with 169K, suggesting that there is some enrichment of residues other than P at position 437 in viruses that have 169E ($P < 0.001$ by Fischer's exact test). This finding is consistent with a number of previous studies that showed a functional interaction between the C4 and V2 regions, resulting in the modulation of neutralizing epitopes in V2 (50–54). The enrichment of mutations at this site in sequences that contain the K169E mutation further suggests an interaction between these two sites. This is further supported by the close proximity of residue 437 in the C4 region to the V2 region within the trimeric form of the envelope. It is still not clear, however, whether C4 forms part of the antibody epitope or plays a more indirect role in modulating the structure of the V2 region, as has been suggested for less broad anti-V2 NAbs that target an epitope overlapping that of CAP256 (51).

Our observation that many of the 39-month viruses remained sensitive to plasma antibodies that arose 2 years earlier, a time point that corresponded to the development of BCN antibodies, indicated the possibility of incomplete viral escape, especially among cluster 1 viruses. The neutralization resistance of the chimeric virus 39mo.C2.V1V2 (a resistant heterologous virus with the 256.39mo.C2 virus V1V2) suggests that this residual neutralization sensitivity can be attributed to an undefined second specificity outside V1V2. Nonetheless, the finding that six of eight 39-month viruses remained sensitive to neutralization by contemporaneous plasma at titers as high as 1:1,280 highlights the possibility that such titers do not play a significant role *in vivo*. This is supported by the finding in other cohorts that superinfection occurred despite preexisting titers against the superinfecting strain that exceeded 1:1,300 (49). Furthermore, estimates of protective titers of antibodies derived from a passive immunization study using neutralizing monoclonal antibodies suggested that in the context of chronic infection (unlike acute infection), titers exceeding 1:1,000 were required to control viremia (55). Perhaps more significantly, the complete absence of contemporaneous neutralization against two of eight 39-month clones highlights that neutralization escape from the CAP256 NAbs was certainly possible. The fact that fully escaped variants were not preferen-

tially selected suggests either that complete escape incurred some fitness cost or that incomplete escape did not incur a similar cost. If this is the case, this may have implications for the use of monoclonal antibodies in passive immunization protocols, particularly in the context of chronic infection.

Interest in the ontogeny of V2 antibodies has intensified since the demonstration that they correlated with protection from HIV infection in the RV144 trial (29). Sieve analysis of break-through infections in RV144 identified immune pressure at position 169 (30), and monoclonal antibodies isolated from RV144 vaccinees were dependent on this residue. Whether the antibodies we describe in CAP256 are related to those in RV144 remains to be determined. The frequency of K169-dependent binding and neutralizing antibodies in natural infection is not known, but our data show that dependence on this residue is not limited to BCN antibodies. Nevertheless, understanding the ontogeny of V2 antibodies and their escape mechanisms could be valuable in improving vaccine design.

Overall, these studies highlight the dynamic relationship between autologous and heterologous specificities and underline the possibility of viral escape even from BCN antibodies targeting conserved epitopes. The role of superinfection, if any, in driving rapid development of BCN responses warrants further study. The consistent targeting of the same epitope by strain-specific and, later, BCN antibodies suggests rapid maturation of these responses to acquire breadth, with implications for vaccine elicitation of such specificities. Thus, isolation and characterization of CAP256 BCN monoclonal antibodies and their precursors will be most informative.

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