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Persistence of VRC01-resistant HIV-1 during antiretroviral therapy

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VRC01, a broadly neutralizing monoclonal antibody (bnmAb), can neutralize a diverse array of HIV-1 isolates by mimicking CD4 binding to the envelope glycoprotein gp120. We have previously demonstrated the presence of VRC01-resistant strains in an HIV-1 infected patient during antiretroviral therapy. Here, we report follow-up studies of two subsequent samples from the same patient. With genetic and phenotypic analysis of over 70 full-length molecular clones of the HIV-1 envelope, we show that VRC01-resistant HIV-1 continued to exist and change in its proportion of the infecting virus during treatment with a highly active antiretroviral therapy. Consistent with our previous observation, the resistant phenotype was associated with a single asparagine residue at position 460 (N460), a potential N-linked glycosylation site in the V5 region. The persistence and continuing evolution of VRC01-resistant HIV-1 *in vivo* presents a great challenge to our future preventative and therapeutic interventions based on VRC01.

HIV-1, VRC01, antibody, resistant

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The entry of human immunodeficiency virus type 1 (HIV-1) into the target cell is dependent on interactions between the viral envelope glycoproteins (gp120 and gp41) and cellular receptor CD4 and coreceptors CCR5 or CXCR4 [1–11]. gp120 contains the CD4-binding site (CD4bs), which although critical for viral entry, is also the viral Achilles' heel for broadly neutralizing monoclonal antibodies (bnmAbs) and polyclonal sera from "elite neutralizers," who display exceptionally strong immune control of viral replication [12–19]. With the recent advent of microculturing and antigen-specific sorting of B cells from "elite neutralizers", researchers have isolated increasing numbers of bnmAbs directed against the CD4bs, including VRC01, VRC03, 3BNC117, 3BNC55, VRC-PG04, and NIH45-46 [12–16, 20,21]. The prototype VRC01, for instance, was isolated from a clade-B-infected individual and neutralized 91% of a diverse panel of pseudotyped viruses [13]. VRC03, a close relative of VRC01, neutralized about 57% of these viruses [13]. More recently isolated CD4bs-directed bnmAbs, in particular 3BNC117, 3BNC55, VRC-PG04, and NIH45-46, have shown similar or even higher potency and breadth than the prototype VRC01 [14–16]. Most of these VRC01-like bnmAbs share similar recognition mechanisms, reflected by their convergence on the recognition of the CD4bs on gp120 [17–19]. Their primary mechanism of neutralization must involve blocking the interaction between the virus and receptor CD4 as they compete for the same general area on HIV-1 gp120, although it is worth noting that there may be

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differences in their mechanisms of blocking the gp120-CD4 receptor interaction [12–26].

However, despite the superior potency and breadth of these bnmAbs, each fails to neutralize a small but significant proportion of pseudotyped viruses in each panel. For instance, VRC01 was unable to neutralize about 10% of the viruses tested [13], and many of those resistant strains were not derived from "elite neutralizers", such as the donor from which VRC01 was initially isolated. The rise of VRC01-resistant viruses in "ordinary" individuals suggests that such variants are either naturally occurring or selected under a VRC01-like antibody response at some point during disease progression. VRC01-resistant strains have also been identified in the VRC01 donor [27]. Only selective archival proviral Env variants remained sensitive, whereas all contemporary plasma-derived variants were resistant, indicating their rapid genetic and phenotypic evolution under strong antibody-based selection pressure [27].

In the course of characterizing diverse HIV-1 strains isolated from infected patients in China, we have identified VRC01-resistant strains derived from CRF08-BC-infected patients [28]. We have also demonstrated that the V5 region, in particular the asparagine residue at position 460 (N460), a potential N-linked glycosylation site (PNGS) in the V5 region, plays a critical role in determining the VRC01-resistant phenotype of these strains [29]. In the present study, we determined the *in vivo* prevalence and evolution patterns of VRC01-resistant variants *in vivo*. With genetic and phenotypic analysis of over 70 full-length molecular clones of the HIV-1 envelope from blood samples taken from a single, previously investigated patient, we show that VRC01-resistant HIV-1 persisted and evolved in both its proportion and sequence during treatment with a highly active antiretroviral therapy. Consistent with our previous observation, the resistant phenotype was associated with the single asparagine residue at position 460 (N460). The persistence and continuous evolution of VRC01-resistant HIV-1 *in vivo* imposes a tremendous challenge for preventative and therapeutic interventions based on VRC01.

1 Materials and methods

1.1 Study subject and PCR amplification of full-length gp160 envelope genes

The study subject is an HIV-1 CRF08_BC-infected intravenous drug user, as reported previously [28]. This patient has received antiretroviral therapy since 2007 and his plasma viral loads were well suppressed (Table 1). His peripheral blood $CD4^+$ T cells increased from 88 cells μL^{-1} on July 26, 2007 to 558 cells μL^{-1} on July 20, 2009 (Table 1). Our initial study was conducted on a sample collected on July 16, 2007 (20070716) and the two samples examined here were collected on January 21 and July 20, 2009 (20090121 and 20090720, respectively), approximately 18 and 24 months later (Table 1). This study was approved by the ethic committees of the appropriate institutions [28].

Table 1 Clinical, virological, and immunological characterization of the subject

Sample points	Treatment	VL (copies mL^{-1})	CD4 counts	CD8 counts
2007-7-26	AZT+3TC+NVP	<50	88	651
2007-10-25	AZT+3TC+NVP	<50	377	651
2008-7-25	AZT+3TC+NVP	<50	572	1826
2009-1-21	AZT+3TC+NVP	<50	376	na
2009-7-20	AZT+3TC+NVP	<50	558	na

Peripheral blood mononuclear cells (PBMC) were collected and genomic DNA was extracted with the QIAamp DNA Blood Mini Kit (Qiagen, Shanghai, China), according to the manufacturer's protocol. Full-length envelope sequence was amplified with nested PCR with HIV-1 subtype-B′C-specific primers, as previously reported [28]. The PCR product was cloned into the pcDNA™3.1 expression vector (Invitrogen, Carlsbad, CA, USA) and verified by sequencing. The full-length gp160 amino acid sequence was analyzed based on a comparison with HIV-1 HXB2.

1.2 Antibodies and soluble CD4

IgG-1 VRC01 antibodies were kindly provided by Dr. John Mascola (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA). Samples of IgG-1 b12 were obtained from the NIH AIDS Research and Reagents Program. IgG-4 ibalizumab was kindly provided by Dr. David D. Ho (Aaron Diamond AIDS Research Center, The Rockefeller University, USA).

1.3 Pseudotyped virus production

The HIV-1 *env* pseudovirus was generated as described previously [28]. Briefly, 293T cells were cotransfected with the pcDNA™3.1 expression vector containing the target *env* gene and the pNL4-3R-E-luciferase viral backbone plasmid in a 1∶3 ratio. The cell culture medium was replaced with 10% Dulbecco's modified Eagle's medium containing fetal bovine serum after 4–6 h and then incubated for an additional 40–48 h at 37°C. The pseudotyped viral supernatants were collected after 48 h. The viral titers were quantified by measuring the luciferase activity in relative light units (RLU; Bright-Glo Luciferase Assay System, Promega Biosciences, CA, USA). The supernatants were packaged and stored at –80°C.

1.4 Analysis of viral sensitivity to neutralization

Neutralization assays were performed as described previously [29]. In brief, 100 $TCID_{50}$ of pseudotyped virus was

incubated with either serially diluted VRC01, b12, or Ibalizumab for 1 h at 37°C before it was used to inoculate TZM-bl cells (approximately 1.5×10^4 cell/well). Infectivity was quantified as luciferase activity (RLU) 48 h after infection. Half-maximal inhibitory concentrations (IC_{50}) are the concentrations required to inhibit infection by 50% compared with the control, and were calculated using the dose-response-inhibition model with a variable slope in GraphPad Prism, version 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

1.5 Sequence and statistical analysis

Sequences were aligned together with selected subtypes/*circulating recombinant form*s of geographic importance using the ClustalW program [30]. A phylogenetic analysis was performed with the neighbor-joining method with the Mega package [31]. The reliability of the branching orders was tested by the bootstrap analysis of 1000 replications [32].

2 Results

2.1 Functional cloning of full-length gp160 envelope genes from patient PBMC

Our laboratory recently characterized the neutralization sensitivity of HIV-1 *env* clones isolated from chronically infected patients in China [28,29]. We identified two CRF08-BC *env* clones (CNE47 and CNE48), isolated at a single time point from the same individual, which displayed substantial differences in their sensitivity to VRC01 neutralization. To study the potential persistence of these variants, we collected two subsequent PBMC samples, approximately 18 and 24 months later, from this individual. The full-length gp160 *env* genes were PCR amplified and cloned directly into a eukaryotic expression vector. A total of 52 and 43 isolates were successfully cloned from sample 20090121 and 20090720, respectively. Pseudotyped viruses were generated by the cotransfection of 293T cells with each of these expression vectors together with a pNL4-3R-E-luciferase viral backbone plasmid. The capacity of the envelope to mediate viral entry was then tested by inoculating TZM-bl cells (approximately 1.5×10^4 cell/well) with the pseudotyped virus and measuring the luciferase activity (RLU) 48 h after infection. As shown in Figure 1, the majority of clones (45/52 from sample 20090121 and 30/43 from sample 20090720) were functional in terms of mediating viral entry, because their luciferase activity was similar to that of the positive control (CNE47). However, the positive clones differed in their entry efficiency, and seven from sample 20090121 and 13 from sample 20090720 failed to facilitate viral entry and their average luciferase activities were well below 1000 RLU (Figure 1).

2.2 Two clusters of envelope sequences were identified in the patient's PBMC

To study the genetic features of these functional clones (45+30=75), we first conducted a sequencing analysis. Among the 45 clones from sample 20090121, 24 were unique, whereas among the 30 clones from sample 20090720, 12 were unique in terms of their nucleotide sequences. A phylogenetic analysis showed that these sequences fell into two major clusters: cluster 1 was closely related with the original CEN47 and CNE48, whereas cluster 2 was quite distant (Figure 2). The intracluster genetic distance was 0.019±0.003 for cluster 1 and 0.005±0.001 for cluster 2, whereas the intercluster distance was 0.039±0.006. Overall, cluster 1 was genetically more closely related to CNE47 and CNE48 (0.034±0.005) than to cluster 2 (0.042±0.007), suggesting that in evolutionary terms, the sequences in cluster 1 shared the same lineage with CNE47 and CNE48, whereas those in cluster 2 belonged to a relatively distant lineage.

Close examination of the phylogenetic tree revealed that all the sequences in cluster 1 were derived from sample 20090121, and all the sequences in cluster 2 were from sample 20090720. Because these two samples were collected only six months apart, their distinct sequence features suggest that the two clusters were probably evolving independently, although the original ancestor of both clusters is uncertain. It must be noted that because the full-length gp160 sequences were amplified directly from PBMC, without prior limiting dilution for single-genome amplification, the exact proportions and their fluctuations in the VRC01-resistant strains relative to the sensitive strains must be interpreted with caution. Furthermore, the sequences in both clusters were genetically more closely related to the prototype reference sequence 08.BC.CN.GX_6F than to any other sequence (Figure 2), consistent with our previous findings.

2.3 Analysis of amino acid variations in the VRC01-binding domain

Structural analysis of VRC01 bound to monomeric gp120 showed that it binds to the CD4bs, largely through loop D, the CD4-binding loop, the base of the V5 loop region, and β24 [33]. To study the evolutionary changes in the amino acid residues in these regions, the full-length gp160 nucleotide sequences were translated into amino acid sequences and aligned with references HXB2, CNE47, and CNE48 (Figure 3). In the hypervariable regions, including V1, V2, V3, V4, and V5, significant differences were found between the sequences from samples 20090121 and 20090720, including both mutational variations and length polymorphisms. This finding supports the phylogenetic analysis, in which the sequences from the samples were separated largely because of these non-silent mutations in the hyper-

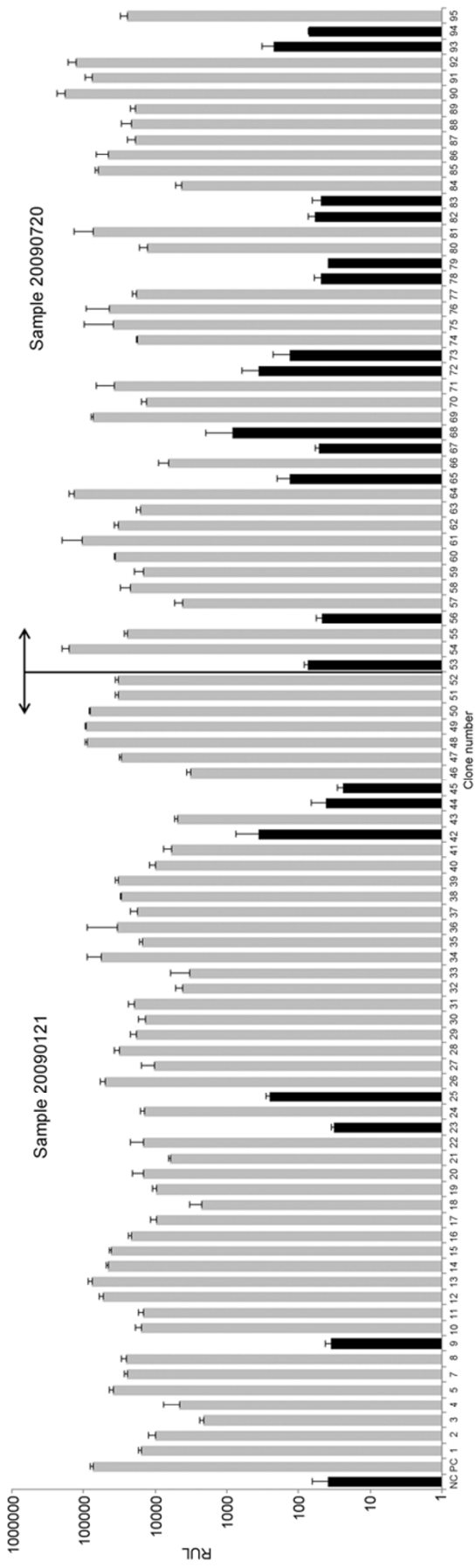

Figure 1 Functional analysis of full-length envelope sequences from two separate PBMC samples (20090121 and 20090720) in the context of pseudotyped viruses. Envelope sequences capable of mediating viral entry (RLU>1000) are highlighted in light gray and those that failed to do so (RLU<1000) are in black. The data are the averages of three independent experiments.

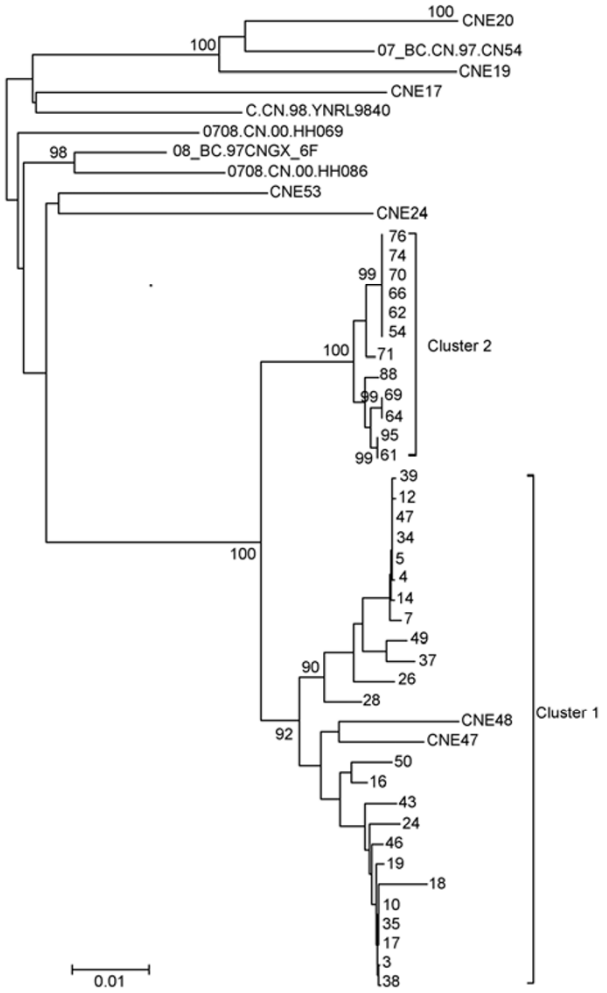

Figure 2 Unrooted neighbor-joining tree showing the genetic relationships among the full-length gp160 envelope clones. The horizontal branch lengths are drawn to scale so that the relatedness of different sequences can be readily assessed. Sequences in two clusters are indicated. A number of commonly used reference sequences for classifying HIV-1 subtypes and *circulating recombinant form*s and several previously characterized variants from China (CNE series) are included. The reliability of the branching order was tested by a bootstrap analysis of 1000 replications and those with 100% confidence are indicated [32].

variable regions. In contrast, loop D and the CD4-binding loop were extremely well conserved (Figure 3). This finding suggests that any changes in viral sensitivity to VRC01 are probably confined to residues within the V5 region rather than involving residues in the other VRC01-binding domains.

2.4 Persistence of VRC01-resistant strains throughout the study period

To study the viral sensitivity to VRC01 neutralization, we generated two representative pseudotyped viruses from the full-length gp160 genes from samples 20090121 and 20090720. These two gp160 genes (#5 and #61) were selected based on their unique V5 regions, because no sequence variations were found in loop D or the CD4-binding loop compared with the original CNE47 and CNE48 (Figure 3). As shown in Figure 4, the pseudotyped viruses built from the two envelopes were as resistant to VRC01 (IC_{50}=15.14 μg mL^{-1} for #5 and IC_{50}=11.75 μg mL^{-1} for #61) as CNE47 (IC_{50}=9.27 μg mL^{-1}) (Table 2). Like the original CNE47 and CNE48, both clones maintained their natural resistance to b12. However, all the pseudotyped viruses were sensitive to neutralization by Ibalizumab (Figure 4, Table 2). Close examination of the V5 amino acid sequences revealed that both clones #5 and #61 contained a single asparagine residue at position 460 (N460), a potential PNGS, which we have previously shown is critical in conferring VRC01 resistance [29]. No other PNGSs were identified in the V5 region of the two clones, although two additional residues (Asn and Glu) were inserted into the V5 region of clone #61 relative to that of #5 (Figure 3, Table 2). Mutational changes from Gln to Lys and Thr at position 458 were found in clones #5 and #61, respectively, and from Thr to Ile at position 467 in clone #61 compared with the original CNE47 sequence. None of these changes seems to have had any significant effect on viral sensitivity to VRC01, because neither #5 nor #61 differs dramatically from CNE47 in terms of their VRC01 sensitivity. Nevertheless, these results suggest that VRC01-resistant viruses persist and increase in their proportion of the infecting viruses in the peripheral blood, despite genetic mutations arising during antiretroviral therapy.

3 Discussion

In this study, we have characterized the evolutionary features of full-length gp160 from an HIV-1-infected individual

Table 2 Neutralization sensitivity to VRC01, b12, and ibalizumab

Clone	Sequence V5/β24 (458–469)	Neutralization					
		VRC01		b12		Ibalizumab	
		IC_{50} (μg mL^{-1})	% of CNE47	IC_{50} (μg mL^{-1})	% of CNE47	IC_{50} (μg mL^{-1})	% of CNE47
CNE47	GGQTNET-NNT-E TFR	9.27	100	>50	100	0.17	100
5	GGKTNET-NNT-E TFR	15.14	163.32	>50	100	0.09	52.94
61	GGTTNSTNNNTEEIFR	11.75	126.75	>50	100	0.17	100

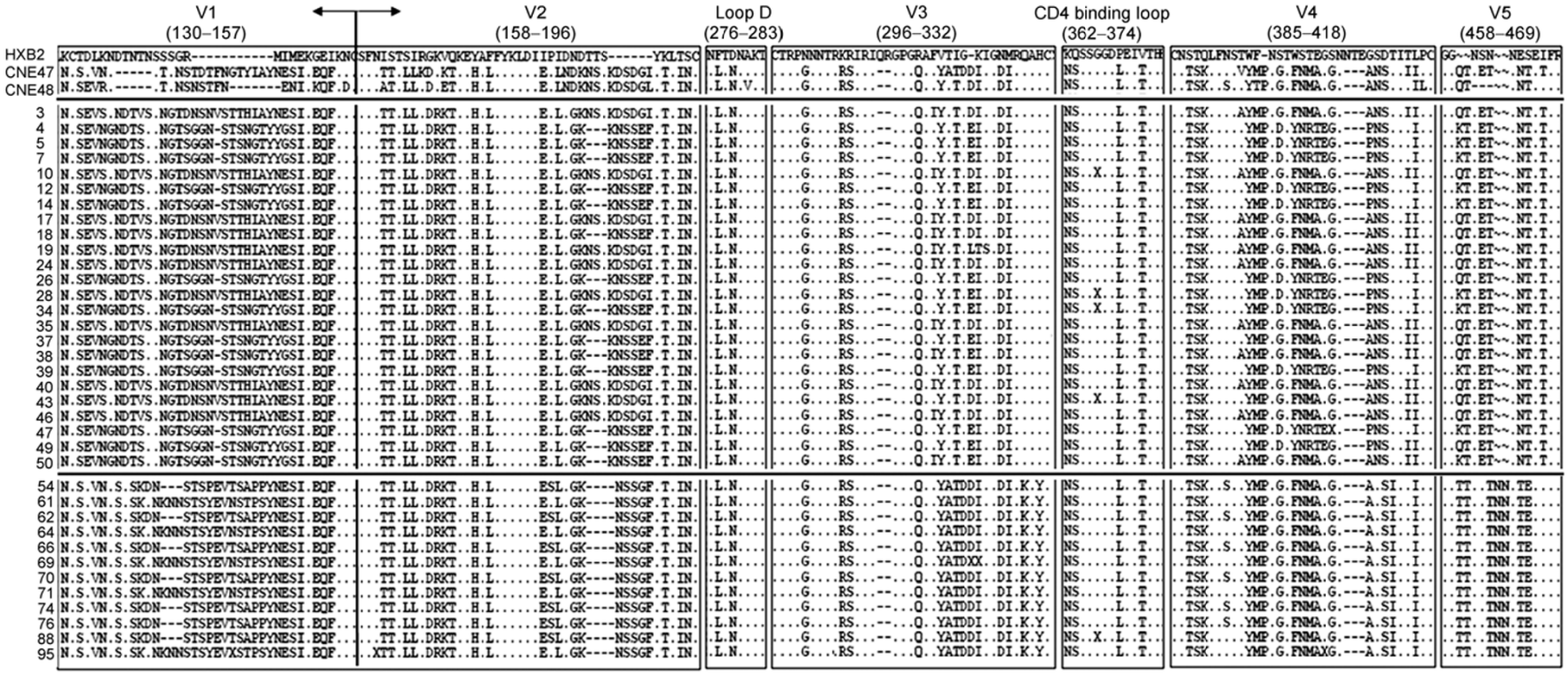

Figure 3 The gp160 amino acid sequences from the study subject are shown and aligned against that of the HXB2 reference strain. Dots represent identical residues, whereas dashes represent gaps introduced to preserve the alignment. Regions known to interact with VRC01, including the CD4-binding loop, loop D, and the V5 region, are highlighted in boxes, as are the hypervariable regions V1, V2, V3, and V4.

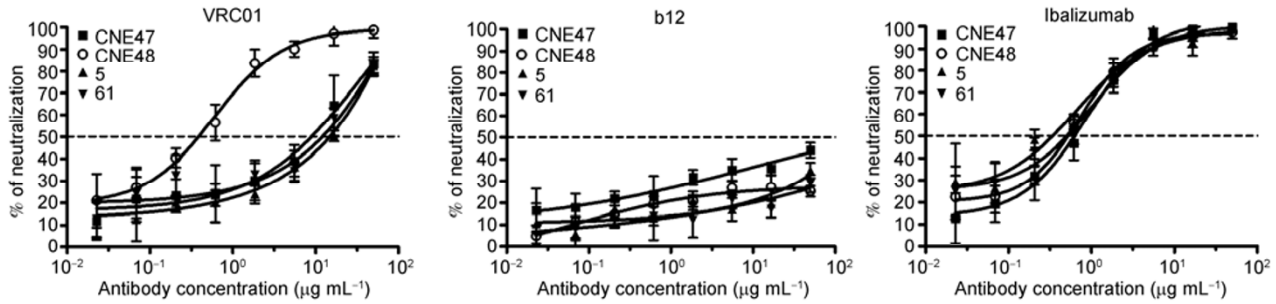

Figure 4 Comparison of representative envelope clones (#5 and #61) derived from mutant viruses with the original CNE47 and CNE48, in terms of their neutralization sensitivities to VRC01, b12, and ibalizumab. Both representative clones showed similar levels of resistance to VRC01 as CNE47. None of these mutations altered their sensitivity to b12 or ibalizumab neutralization.

whom we had previously shown to harbor VRC01-resistant strains. A genetic analysis identified significant changes in the hypervariable regions of gp160 during the study period, including both mutational variations and length polymorphisms. Phenotypic characterization of representative gp160 clones showed the persistence of the VRC01-resistant phenotype during the course of antiretroviral therapy. As in the original CNE47, the resistant phenotype was strongly associated with N460, a potential PNGS in the V5 region. The persistence and continuous evolution of VRC01-resistant HIV-1 *in vivo* will pose challenges to our future preventative and therapeutic interventions based on VRC01.

This is not the first report of VRC01-resistant strains of HIV-1. However, the mechanisms underlying the generation of these strains remain poorly understood, as do their evolutionary mechanisms *in vivo*. Although we have shown that N460 in the V5 region is critical for viral sensitivity to VRC01 in CRF08-BC-infected patients, we cannot exclude the possibility that other factors also confer resistance to VRC01. Further research is required to determine the impact of these V5 mutations on VRC01 sensitivity in a broader range of viral strains, especially viruses isolated from patients with VRC01-like neutralization activity. Furthermore, because many infected individuals do not show a detectable VRC01-like antibody response, many VRC01-resistant viral variants are probably generated by random genetic mutations, and once they are generated, their persistence or turnover will be dependent on factors other than VRC01-like antibody responses. It is unlikely that their persistence in this individual was directly related to his antiretroviral therapy because the viral loads in the plasma were well suppressed and there was no rebound of resistant viruses. Alternatively, if a VRC01-like antibody response did exist at some period during infection, the generation and turnover of VRC01-resistant strains would then be largely dependent on the magnitude and duration of that response. In either case, the sheer presence and persistence of VRC01-resistant HIV-1 will pose tremendous challenges to anti-HIV-1 strategies based on VRC01. Strategies involving other bnmAbs, together with VRC01, will probably be more efficacious than those based on VRC01 alone.

Efforts are being made to develop novel immunogens and immunization strategies that can induce potent anti-CD4bs antibodies [34–40]. VRC01 remains one of the leading mAbs for HIV-1 prevention research and development, but the natural emergence of HIV-1 resistance has caused uncertainty about its broader use. Nonetheless, detailed analyses of VRC01 escape mechanisms and patterns of evolution increase our understanding of the interactions between the virus and the immune system *in vivo*, so that we can better target strategies to address treatment resistance. More studies are required to fully understand how the virus circumvents neutralization, particularly analyses of VRC01 sensitivity in a broader range of viral strains and their associated biological properties. Such research will undoubtedly optimize the development process and maximize the potential use of VRC01 in the clinical setting.

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