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Sequences in Glycoprotein gp41, the CD4 Binding Site, and the V2 Domain Regulate Sensitivity and Resistance of HIV-1 to Broadly Neutralizing Antibodies

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The swarm of quasispecies that evolves in each HIV-1-infected individual represents a source of closely related Env protein variants that can be used to explore various aspects of HIV-1 biology. In this study, we made use of these variants to identify mutations that confer sensitivity and resistance to the broadly neutralizing antibodies found in the sera of selected HIV-1-infected individuals. For these studies, libraries of Env proteins were cloned from infected subjects and screened for infectivity and neutralization sensitivity. The nucleotide sequences of the Env proteins were then compared for pairs of neutralization-sensitive and -resistant viruses. *In vitro* mutagenesis was used to identify the specific amino acids responsible for the neutralization phenotype. All of the mutations altering neutralization sensitivity/resistance appeared to induce conformational changes that simultaneously enhanced the exposure of two or more epitopes located in different regions of gp160. These mutations appeared to occur at unique positions required to maintain the quaternary structure of the gp160 trimer, as well as conformational masking of epitopes targeted by neutralizing antibodies. Our results show that sequences in gp41, the CD4 binding site, and the V2 domain all have the ability to act as global regulators of neutralization sensitivity. Our results also suggest that neutralization assays designed to support the development of vaccines and therapeutics targeting the HIV-1 Env protein should consider virus variation within individuals as well as virus variation between individuals.

It has been known for many years that sera from approximately 30% of HIV-1-infected subjects possess broadly neutralizing antibodies (bNAbs) able to neutralize viruses from multiple strains of HIV-1 (9, 10, 34, 52, 56, 58, 65). A major hypothesis in HIV-1 vaccine research has been that a vaccine able to elicit bNAbs such as those found in HIV-infected sera would be effective at preventing HIV transmission (1, 5). However, HIV-1 employs many mechanisms to evade the immune response, and there is concern that resistance to neutralizing antibodies will be as great a challenge for HIV-1 vaccines as drug resistance is for HIV-1 therapeutics. In this and previous studies (42, 43), we adapted a technology first developed to characterize drug resistance (15, 46, 49, 54) for the purpose of characterizing polymorphisms that confer sensitivity and resistance to bNAbs. This technology, swarm analysis, allowed us to investigate the molecular determinants of neutralization sensitivity and resistance to bNAbs in HIV-infected polyclonal sera and to define the specificity of these antibodies.

Swarm analysis makes use of the large number of naturally occurring, and closely related, envelope (Env) variants that arise in each HIV-1-infected individual. In particular, the sequences of neutralization-sensitive and neutralization-resistant viruses arising in the same individual are compared, and *in vitro* mutagenesis is used to identify the specific polymorphisms that determine the neutralization phenotype. In previous studies, we used this technology to identify mutations in clade B viruses that appeared to increase neutralization sensitivity by overcoming conformational masking and thus enhancing the exposure, simultaneously, of multiple epitopes recognized by broadly neutralizing monoclonal antibodies (bN-MAbs) and viral entry inhibitors. One of the mutations occurred at position 653 (HXB2 numbering) in the C34 helix of gp41 and appeared to destabilize the six-helix bundle essential for virus fusion (43). The other mutation occurred in the V2 domain of gp120, at position 180, in a sequence that appears to occur at the apex of the gp160 trimer and includes a binding site for the α4β7 integrin (42). In the present study, we analyzed the virus quasispecies from five different individuals who became infected with clade B viruses while participating in a phase 3 HIV-1 vaccine trial (14). We report additional mutations that alter neutralization sensitivity/resistance. Our results demonstrate that polymorphisms in gp41 and the CD4 binding site, as well as in the V2 domain, play a major role in determining neutralization sensitivity and resistance. These results extend our knowledge of the relationship between the structure and function of the HIV-1 Env protein, as well as the knowledge of molecular determinants of neutralization sensitivity and resistance.

MATERIALS AND METHODS

Envelope genes and swarm analysis. Libraries of full-length Env genes were isolated from cryopreserved plasmas from 57 subjects who became infected with HIV-1 while participating in a phase 3 trial (14) of a candidate HIV-1 vaccine (AIDSVAX B/B) (1). The specimens selected for analysis were provided by Global Solutions for Infectious Diseases (GSID) (South San Francisco, CA) and represented the earliest postinfection sam-

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ples available, with an estimated mean time after infection of 109 ± 58 days (45). Libraries representing the Env variants from the swarm of quasispecies that evolved in each infection were constructed at Monogram Biosciences (South San Francisco, CA) by PCR amplification and then cloned into the expression vector pCXAS-PXMX as described previously (42, 43, 66). The protocol used for isolation of Env genes and the subsequent selection and characterization of individual clones has been described previously (15, 49, 54). Single Env clones were then selected for the production of pseudotype viruses, which were then screened for infectivity and receptor tropism in U87 cells that expressed CD4, CCR5, and CXCR4 ($CD4^+$ $CCR5^+$ $CXCR4^+$ U87 cells) (66).

Ten to 12 Env genes were selected for each individual and evaluated in virus neutralization assays. For each individual, the two clones exhibiting the greatest difference in neutralization sensitivity, as measured with a panel of three HIV-positive sera with bNAbs, were selected for further study. These were designated wild-type sensitive (wtS) and wild-type resistant (wtR) clones.

Antibodies and antiviral drugs. Three clade B HIV-positive sera containing bNAbs (Z23, N16, and Z1679) were provided by Monogram Biosciences. These are known from previous studies (42, 43, 54) to neutralize a wide variety of primary clinical isolates of HIV-1 (see Table S1 in the supplemental material). A panel of bN-MAbs was assembled from various sources. These MAbs included b12 (6) and 4E10 (59), obtained from Polymun AG (Vienna, Austria). The bN-MAbs PG9 and PG16, described by Walker et al. (63), were obtained from the International AIDS Vaccine Initiative (New York, NY). The 17b and 48d MAbs, which bind preferentially to the CD4-induced (CDi) conformation of gp120 (39, 60), were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and directly from James E. Robinson (Tulane University, New Orleans, LA). The VRC01 MAb, directed to the CD4 binding site (67), was obtained through the NIH AIDS Research and Reference Reagent Program.

Virus neutralization assay. A high-throughput virus neutralization assay was used to measure the ability of MAbs and antibodies in HIV-1-positive plasma to inhibit the infectivity of pseudotype viruses (7, 46, 49, 54). Briefly, pseudotype viruses were prepared by cotransfecting HEK293 cells (American Type Culture Collection [ATCC], Manassas, VA) with an Env expression vector and an Env-deficient HIV-1 genomic vector carrying a luciferase reporter gene. Serial dilutions of MAbs or plasmas/sera were incubated with pseudotype viruses for 1 h prior to addition of U87 cells expressing CD4, CCR5, and/or CXCR4. The Z23 serum was used at an initial dilution of 1/100 and was included as an internal control in all experiments.

In preliminary studies, the N16 and Z1679 sera were used at a starting dilution of 1/20 or 1/40. However, this was reduced to 1/100 to conserve serum in subsequent experiments. Neutralization data are reported as 50% inhibitory concentrations (IC_{50}s) calculated from serum dilution curves. The virus controls included pseudoviruses prepared from the neutralization-sensitive HIV-1 isolate NL4-3 and the less neutralization-sensitive primary isolate JRCSF. The negative-control virus consisted of pseudotype viruses prepared from the envelope of the amphotropic murine leukemia virus (aMLV). HIV-1 neutralization titers were considered significant only if they were >3 times higher than the aMLV titers. The neutralization assays carried out at Monogram Biosciences were performed according to good laboratory practice (GLP) and using protocols approved under Clinical Laboratory Improvement Amendments (CLIA). Each assay included acceptability criteria to ensure that interassay variation between IC_{50}s, measured with reference standards, fell within 2.5-fold 95% of the time.

Sequencing, mutagenesis, and bioinformatic analysis. Plasmids containing cloned Env glycoproteins were sequenced using Sanger sequencing at either Monogram Biosciences or the University of California Sequencing Facility (Berkeley, CA). Mutations were introduced into HIV-1 Env proteins by site-directed mutagenesis using a QuikChange Lightning kit (Agilent, Santa Clara, CA), followed by confirmatory sequencing. HXB2 numbering by alignment with sequence AF033819 from the GenBank database is provided throughout. Visualization of the Protein Data Bank (PDB) coordinates was performed using PyMol (8), and glycans were modeled using Glyprot (4).

Nucleotide sequence accession numbers. The sequences of the five pairs of neutralization-sensitive and -resistant Env genes described in this study have been deposited in GenBank under accession numbers JQ085287 to JQ085296.

RESULTS

In previous studies, we used swarm analysis to identify mutations in two subjects from the VAX004 trial that resulted in differences in sensitivity to neutralization by bNAbs in sera from HIV-infected subjects (42, 43). In this study, we identified mutations conferring neutralization sensitivity/resistance in five additional subjects. Of the 57 subjects from the VAX004 trial (14) who have been screened to date, 12 exhibited pairs of neutralization-sensitive and -resistant viruses suitable for swarm analysis. The specimens used for swarm analysis were selected on the basis of being from recent infections (mean time after infection, 109 ± 58 days), without regard for whether they were from vaccine or placebo groups. Because there was no evidence of vaccine efficacy in this trial and because no differences were detected between vaccinees and placebo recipients with respect to virus loads, CD4 counts, time to initiation of antiretroviral therapy (14, 17), or phylogenetic differences in Env sequences (45), samples from both groups were selected at random for use in these studies.

To identify specimens appropriate for swarm analysis, 24 to 48 Env clones were isolated from each individual and screened for infectivity. Ten to 12 clones with high infectivity were then tested for sensitivity/resistance to a panel of three HIV-infected sera, i.e., Z23, N16, and Z1679, all known to possess bNAbs. We then selected pairs of viruses from five individuals that exhibited at least a 3-fold difference in sensitivity/resistance to neutralization by one or more of the HIV-infected sera. The results of clonal analysis studies to identify neutralization-sensitive and -resistant pairs of viruses from the subjects are provided in Table 1. For each subject, we designated one clone as wild type and neutralization resistant (wtR) and another clone as wild type and neutralization sensitive (wtS). The difference in sensitivity between pairs of envelopes from the same individual varied in magnitude from 3- to over 100-fold. When the sequences of the wtR viruses from the individuals were compared to the sequences of the wtS viruses, we found that the most divergent clonal pair (from donor 108069) differed at 20 amino acid positions, whereas there were only 6 amino acid differences between the pair of envelopes from donor 172946 (Table 2). Site-directed mutagenesis was then used to systematically replace individual amino acids in the resistant clone with the corresponding amino acids from the sensitive clone. The resulting envelopes were used to create pseudotype viruses that were then tested for neutralization sensitivity/resistance with the original HIV-infected sera. A listing of the sequence differences between the neutralization-sensitive and -resistant viruses is provided in Table 2. The locations of the sequence differences in the linear gp160 sequence are provided in Fig. 1.

Single amino acid substitutions in the V2 domain and the V2 stem confer sensitivity to bNAbs in HIV sera. Of the five subjects studied, two possessed envelopes with single amino acid differences that conferred neutralization sensitivity to a neutralization-resistant backbone. In the case of subject 172950, we observed an

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TABLE 1 Identification of pairs of Env clones that differ in sensitivity/resistance to broadly neutralizing antibodies in HIV-positive sera[a]

Subject or virus	Clone	Neutralization titer (IC_{50}) of HIV-positive serum (1/dilution)		
		Z23	N16	Z1679
172950	006	**236**	49	**245**
	007 wtR	**208**	38	**166**
	010	**327**	**91**	**420**
	029	**635**	**128**	**1,158**
	032	**313**	**83**	**277**
	036 wtS	**2,488**	**838**	**4,406**
	040	**793**	**194**	**803**
	041	**326**	**86**	**369**
172976	003	**126**	29	**60**
	015	**152**	32	**84**
	032 wtR	<100	21	<20
	033	**205**	28	**52**
	034	**180**	29	**80**
	035 wtS	**2,118**	**314**	**1,373**
	042	**269**	24	28
	043	<100	24	<20
172297	005	**360**	**44**	**229**
	013 wtR	**396**	**28**	**208**
	018	**517**	**41**	**284**
	019	**480**	<20	**420**
	025	**567**	**56**	**371**
	027	**1,385**	**166**	**1,076**
	028 wtS	**11,414**	**2,897**	**7,473**
	030	**1,680**	**92**	**956**
	037	**706**	**66**	**353**
	046	**13,767**	**2,705**	**5,351**
172946	001	**281**	**75**	**154**
	008 wtR	107	21	**152**
	011	**312**	**102**	**248**
	012	199	**52**	**173**
	021	125	**37**	**310**
	024	**458**	**75**	**844**
	025 wtS	**5,183**	**1,710**	**4,336**
	037	**440**	**60**	**902**
	039	**237**	**80**	**233**
108069	002	**335**	46	75
	005 wtR	171	<40	65
	006	**446**	**113**	159
	007	**324**	63	124
	009	**182**	<40	71
	010	**326**	**63**	86
	011 wtS	**393**	**151**	**262**
	012	**314**	50	61
	018	**181**	<40	70
	022	**323**	44	61
JRCSF		**323**	**161**	**245**
		385	**177**	**486**
NL4-3		**2,526**	**1,162**	**1,791**
		3,598	**769**	**1,803**
aMLV		58	<40	66
		<100	<20	<20

[a] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition of target cell infection. Values in bold represent significant neutralization titers that are at least three times greater than those observed against the negative control (aMLV). The clade B NL4-3 and JRCSF viruses were included as CXCR4- and CCR5-dependent positive controls, respectively. All clones tested were CCR5 tropic. wtR, wild type and resistant; wtS, wild type and sensitive.

TABLE 2 Summary of amino acid sequence differences between neutralization-sensitive and -resistant clones[a]

Subject	wtR clone	wtS clone	Amino acid differences
172950	007	036	**N197H**, T257A, V281G, R304G, A316V, T436A, S440R, R707K, A833V
172976	032	033	**D167G**, R282K, I285L, R335G, ΔN460, I686 M, F833L
172297	013	028	T60A, N136K, L345I, **Y384H**, D460N, S465N, V535A, **L702P**
172946	008	029	K92E, S142G, **I423T**, **G589D**, R696K, F765L
108069	005	011	H105Q, G263S, Y330H, N407Y, Y409N, ΔK410, E411T, K412G, R440S, I447S, N464D, A612T, E630Q, R656K, **S668G**, **I675V**, V817A, A820I, I832V, A836I

[a] Differences shown in bold indicate amino acid polymorphisms required in each clone pair to restore a neutralization-sensitive phenotype to the neutralization-resistant clone. All clones were CCR5 tropic, with the exception of 108069_005_S668G, which had a dual CCR5/CXCR4 usage.

8- to 12-fold difference in neutralization titer between the wtS clone, 036, and the wtR clone, 007 (Table 1). When the amino acid sequences were compared, there were nine differences between the two clones (Table 2). The differences were located in the V2 stem, C2, V3, and C4 domains of gp120, as well as the cytoplasmic tail of gp41 (Fig. 1). Sequential mutagenesis (Table 3) showed that replacement of the asparagine (N) at position 197 with a histidine (H) (N197H) was able to convert neutralization-resistant clone 007 into a neutralization-sensitive envelope with properties similar to those of the wtS clone, 036. Loss of asparagine at position 197 resulted in the destruction of a predicted N-linked glycosylation site (PNGS). To determine if the polymorphism at position 197 affected a single epitope or multiple epitopes, studies were carried out with bN-MAbs to the V2 domain, the V3 domain, the CD4 binding site, and the membrane-proximal external domain (MPER) in gp41 (Table 4). The wtR clone was resistant to neutralization by the b12, PG16, and 4E10 MAbs but showed weak sensitivity to neutralization by the PG9 MAb. When the N197H mutation was incorporated into wtR clone 007, the resulting virus was at least 300-fold more sensitive to neutralization by the b12 MAb (directed to the CD4 binding site), 30-fold more sensitive to CD4 IgG, and at least 6-fold more sensitive to the 4E10 MAb, which binds to the MPER. There was no significant change in neutralization sensitivity observed with the VRC01 MAb, which is able to neutralize a wide range of clinical isolates and is directed to the CD4 binding site (67). The 48d and 17b MAbs preferentially bind to the CD4i conformation and are able to neutralize lab-adapted isolates but not primary clinical isolates of HIV-1 (31, 55, 70). There was a trend for increased neutralization by 17b of the 172950 isolate with the N197H mutation, but this did not reach 50% inhibition at the highest concentration (20 μg/ml) tested (see Fig. S1 in the supplemental material). The fact that 48d did not neutralize any of the Envs tested in this study was not unexpected in view of its weak neutralizing activity. The deletion of the glycosylation site at position 197 in the envelope from subject 172950 coincided with increased neutralization by MAbs recognizing several spatially distinct sites. Interestingly, the N197H mutation increased rather than decreased the resistance to the PG9 MAb,

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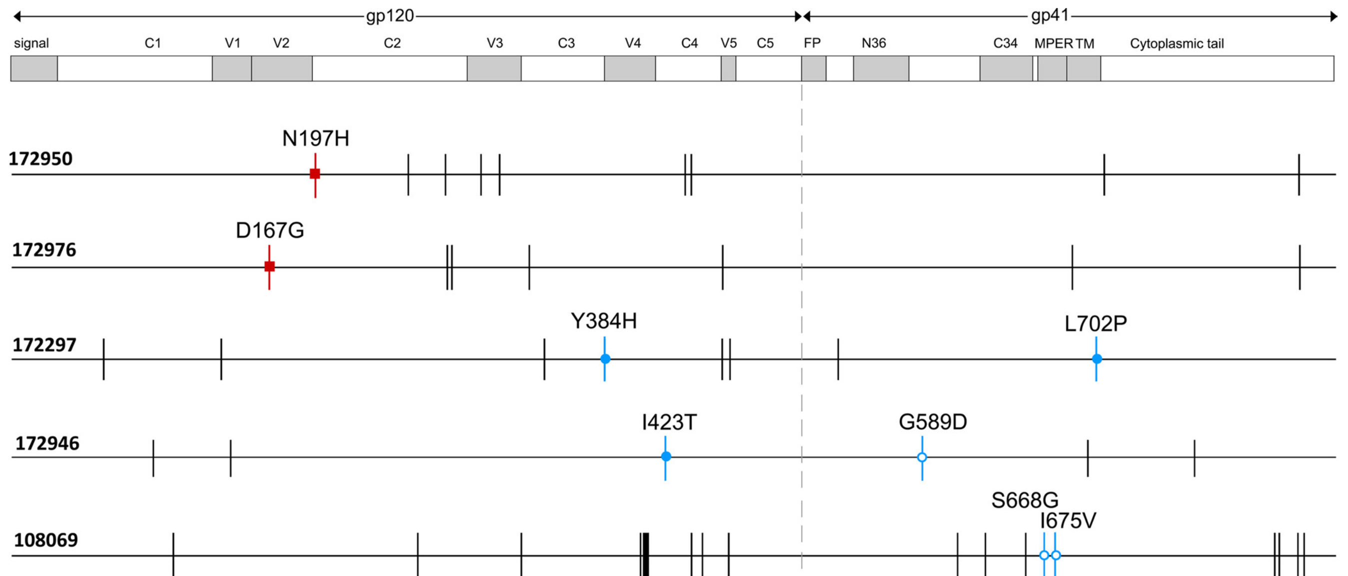

FIG 1 Locations of sequence differences between neutralization-sensitive and -resistant viruses. Each line indicates the location of an amino acid difference between a pair of neutralization-sensitive and -resistant clones isolated from the same individual. A diagram of the major domains of the HIV Env protein is provided at the top of the figure, indicating the locations of conserved (C) and variable (V) domains. Also shown are the locations of the signal sequence (signal), the gp41 fusion peptide, the N36 and C34 helices of gp41, the membrane-proximal external domain (MPER), the transmembrane domain (TM), and the cytoplasmic tail. Closed symbols indicate mutations that are able to independently confer neutralization sensitivity/resistance. Open symbols indicate mutations that depend on a mutation at another site to alter neutralization sensitivity/resistance. Blue symbols represent pairs of mutations involving at least one mutation in gp41 responsible for the neutralization phenotype. Red symbols indicate single mutations in the V2 domain responsible for the neutralization phenotype.

TABLE 3 Mutational analysis to locate amino acid residues responsible for differences in sensitivity/resistance to neutralization by HIV-positive sera possessing broadly neutralizing antibodies[a]

Subject or virus	Clone	Phenotype or mutation	Neutralizing antibody titer (IC_{50}) for indicated human HIV-positive serum sample		
			Z23	N16	Z1679
172950	007	wtR	**214**	<100	<100
	036	wtS	**1,622**	**461**	**1,231**
	007	N197H	**2,480**	**1,114**	**1,573**
	007	T257A/V281G	**627**	**150**	**412**
	007	R304G/A316V	**402**	<100	164
	007	T436A/S440R	<100	<100	<100
	007	R707K/A833V	**180**	<100	<100
172976	032	wtR	<100	<100	<100
	035	wtS	**871**	**339**	**558**
	032	D167G	**475**	**151**	**376**
	032	R282K/I285L	<100	<100	<100
	032	R335G	**126**	<100	<100
	032	ΔN460	<100	<100	<100
	032	I686M	**122**	<100	<100
	032	F833L	<100	<100	<100
JRCSF			**337**	**196**	**164**
NL4-3			**3,708**	**1,543**	**3,701**
aMLV			<100	<100	<100

[a] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition of target cell infection. Values in bold represent significant neutralization titers that are at least three times greater than those observed against the negative control (aMLV). All clones tested were CCR5 tropic. wtR, wild type and resistant; wtS, wild type and sensitive.

which is known to recognize carbohydrate epitopes at positions 156 and 160 (38).

We next examined a pair of viruses from subject 172976 (Table 3), in which case a small but reproducible difference (3- to 8-fold) in sensitivity to neutralization was observed between the wtS clone, 035, and the wtR clone, 032 (Table 1). When the amino acid sequences of these two clones were compared, seven amino acid differences were found (Table 2). These differences were distributed throughout the V2, C2, C3, and V5 domains of gp120, as well as in the MPER and cytoplasmic tail (CT) of gp41 (Fig. 1). Sequential replacement of the amino acids at each position showed that most of the difference in neutralization sensitivity could be attributed to position 167 in the V2 domain, where aspartic acid (D) was replaced by glycine (G) (Table 3). Although Env genes from subject 172976 possessed the canonical motif required for the binding of the PG9 and PG16 MAbs (PNGS at positions 156 and 160 and lysine [K] at position 168), all of the clones from this individual were resistant to neutralization by these antibodies at 10 μg/ml. When the wtR clone and the D167G mutant clone were compared (Table 4), we found equivalent resistances to PG9, PG16, and b12 and no change in sensitivity to the 4E10 or VRC01 MAb. However, the D167G mutation resulted in a 55-fold increase in neutralization sensitivity to CD4 IgG and a small increase in sensitivity to 17b, just above the 20-μg/ml cutoff (Table 4; see Fig. S1 in the supplemental material). Thus, this mutation in the V2 domain appears to increase the exposure or alter the conformation of the CD4 binding site. Previous studies have reported that mutation or deletion of the V2 domain can increase sensitivity to MAbs against the V2 domain and to CD4 IgG (41, 48, 53, 57).

Double amino acid substitutions that confer neutralization by targeting the CD4 binding site and gp41. In each of the remaining three subjects, two or more amino acid changes were

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TABLE 4 Effects of amino acid substitutions on neutralization by monoclonal antibodies, entry inhibitors, and HIV-positive sera possessing neutralizing antibodies[a]

Subject	Clone	Phenotype or mutation	Neutralizing antibody titer (IC_{50} [μg/ml])								
			b12	PG9	PG16	4E10	CD4 IgG	17b	VRC01	48d	Z23[b]
172950	007	wtR	>20	**4.25**	>10	>20	**1.43**	>20	**0.44**	>20	**199**
172950	007	N197H	**0.07**	>10	>10	**3.3**	**0.05**	>20	**0.25**	>20	**1,393**
172976	032	wtR	>20	>10	>10	**1.1,871**	**14.2**	>20	**6.27**	>20	<100
172976	032	D167G	>20	>10	>10	**0.83**	**0.23**	>20	**6.41**	>20	**359**
172297	013	wtR	>20	**0.48**	**0.06**	**8.35**	>20	>20	**0.19**	>20	**223**
172297	013	Y384H/L702P	**0.14**	**0.30**	**0.02**	**0.39**	**0.18**	**1.60**	**0.13**	>20	**7,049**
172946	008	wtR	>20	**5.50**	**2.35**	**10.61**	>20	>20	**0.70**	>20	**178**
172946	008	I423T/G589D	**6.25**	>10	>10	**1.67**	**0.06**	**17.2**	**2.50**	>20	**2,795**
108069	005	wtR	>20	>10	**0.26**	**7.21**	>20	>20	**1.23**	>20	**111**
108069	005	S668G/I675V	>20	>10	**0.48**	**0.11**	**2.0**	>20	**1.12**	>20	**242**
JRCF			**0.18**	**0.01**	**0.01**	**6.01**	**5.1**	>20	**0.36**	>20	**358**
NL4-3			**0.10**	**1.15**	**0.01**	**5.00**	**0.01**	**1.3**	**0.33**	>20	**4,416**
aMLV			>20	>10	>10	>20	>20	>20	>20	>20	<100

[a] Values in bold represent significant neutralization titers that are at least three times greater than those observed against the negative control (aMLV).
[b] Z23 is a reference serum possessing broadly neutralizing antibodies. The data shown for this serum represent the reciprocal of the dilution required for 50% neutralization.

required to convert neutralization-resistant envelopes to neutralization-sensitive envelopes. When viruses from subject 172297 were tested for sensitivity to the panel of HIV-infected sera, we found a substantial (31- to 53-fold) difference in neutralization sensitivity between the wtR clone, 013, and the wtS clone, 028 (Table 1). Comparison of the sequence differences between these clones (Table 2) showed that there were 8 amino acid sequence differences. These differences occurred in the C1, V1, C3, and V5 domains of gp120, adjacent to the fusion domain (FD) in gp41, and in the transmembrane (TM) domain of gp41 (Fig. 1). When single amino acid substitutions were examined (Table 5), we found that the replacement of tyrosine (Y) by histidine (H) at position 384 in the C3 domain of the neutralization-resistant clone 013 resulted in a 6- to 13-fold increase in neutralization sensitivity. In addition, we found that replacement of leucine (L) by proline (P) at position 702 in the TM domain of gp41 also resulted in a 6- to 10-fold increase in sensitivity to neutralization (Table 5). Thus, conversion of the neutralization-resistant clone 013 Env to the extremely neutralization-sensitive clone 028 phenotype required simultaneous mutations at position 384 in the CD4 binding site of gp120 and at position 702, which is thought to be buried in the viral membrane (21, 22, 28). It was not possible to say definitively whether this was an additive effect of two individual mutations or a synergistic effect. However, it was remarkable that a mutation in the transmembrane domain of gp41 appeared to induce a conformational change that affected the binding of neutralizing antibodies to the outer gp120 domain of the HIV-1 Env protein. Studies with MAbs (Table 4) showed that wtR clone 013 was sensitive to neutralization by PG9 and PG16, directed to the V2 domain, and moderately sensitive to neutralization by the 4E10 MAb, directed to the MPER. Clone 013 was also resistant to neutralization by CD4 IgG and the b12 MAb (directed to the CD4 binding site). Introduction of the Y384H and L702P mutations had no effect on neutralization by the PG9 and PG16 MAbs. However, introduction of the Y384H and L702P mutations increased neutralization sensitivity to the 4E10 MAb against the MPER by 21-fold and increased neutralization sensitivity to CD4 IgG and the b12 MAb by at least 109- and 143-fold, respectively. No difference in VRC01-mediated neutralization was observed. Although clone 172297 was resistant to 17b-mediated neutralization, the double mutant 172297_Y384H/L702P was extremely sensitive to the 17b MAb, with an IC_{50} of 1.6 μg/ml, which is 50 times more sensitive than the majority of tier 1A envelopes assayed by Seaman et al. (55) (Table 4; see Fig. S1 in the supplemental material).

We next examined the pair of neutralization-sensitive and -resistant viruses from subject 172946. In this case, there was a 16- to 31-fold difference in sensitivity to neutralization by the panel of

TABLE 5 Mutational analysis to locate amino acid residues responsible for differences in sensitivity/resistance to neutralization by HIV-positive sera possessing broadly neutralizing antibodies[a]

Subject or virus	Clone	Phenotype or mutation	Neutralization titer (IC_{50}) of HIV-positive serum (1/dilution)		
			Z23	N16	Z1679
172297	013	wtR	**230**	40	**133**
	028	wtS	**12,161**	**1,839**	**4,124**
	013	Y384H/L702P	**10,285**	**1,304**	**2,736**
	013	T60A/N136K	**267**	<100	**153**
	013	Y384H	**3,219**	**480**	**793**
	013	L702P	**2,089**	**428**	**787**
	013	D460N/S465N	**298**	<100	<100
	013	L345I	**417**	**51**	**208**
	013	V535A	**519**	<100	**241**
172946	008	wtR	**198**	**52**	**167**
	025	wtS	**6,245**	**1,557**	**2,671**
	008	I423T/G589D	**6,434**	**1,202**	**2,224**
	008	I423T	**1,275**	**200**	**561**
	008	G589D	**128**	<100	**119**
	008	K92E/S142G	**258**	43	**172**
	008	R696K/F765L	**184**	<100	**133**
JRCSF			**337**	**196**	**164**
NL4-3			**3,708**	**1,543**	**3,701**
aMLV			<100	<100	<100

[a] Refer to the footnote to Table 3 for details.

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TABLE 6 Mutational analysis to locate amino acid residues responsible for differences in sensitivity/resistance to neutralization by HIV-positive sera possessing broadly neutralizing antibodies[a]

Subject or virus	Clone	Phenotype or mutation	Neutralization titer (IC_{50}) of HIV-positive serum (1/dilution)		
			Z23	N16	Z1679
108069	005	wtR	**196**	40	<20
	011	wtS	**581**	**161**	**120**
	005	H105Q	**214**	45	45
	005	G263S	**252**	38	38
	005	Y330H	**263**	48	29
	005	N407Y/Y409N/ΔK410/E411T/K412G	**267**	25	37
	005	R440S/I447S	**314**	45	44
	005	N464D	**215**	23	40
	005	A612T	**192**	33	<20
	005	E630Q	**259**	<20	38
	005	S668G	**258**	58	30
	005	I675V	**342**	45	**103**
	005	S668G/I675V	**477**	62	**323**
	005	V817/A820/I832V/A836I	**196**	49	<20
JRCSF			**358**	**269**	**273**
NL4-3			**4,416**	**1,431**	**3,155**
aMLV			<100	<20	<20

[a] Refer to the footnote to Table 3 for details.

HIV-infected sera between the wtS clone, 025, and the wtR clone, 008 (Table 1). Comparison of the predicted amino acid sequences showed that there were six amino acid differences between these two clones (Table 2), distributed in the C1, V1, and C4 domains of gp120, in close proximity to the N36 helix of gp41, and in the TM and CT domains of gp41 (Fig. 1). Analysis of single amino acid substitutions showed that the replacement of isoleucine (I) by threonine (T) at position 423 in the C4 domain (I423T) of gp120 resulted in a modest (3- to 6-fold) increase in neutralization sensitivity to the broadly neutralizing polyclonal antibody panel (Table 5). Interestingly, neither the single G589D mutant nor the two double mutants (K92E/S142G and R696K/F765L) showed a significant increase in neutralization sensitivity. However, when the I423T mutation in C4 was paired with the G589D mutation in gp41, the neutralization-sensitive phenotype was fully restored (Table 5). Studies with MAbs (Table 4) showed a moderate (3-fold) increase in b12 sensitivity associated with the I423T/G589D mutant, a similar moderate (6-fold) increase in 4E10 sensitivity, and a 345-fold increase in CD4 IgG sensitivity. The I423T/G589D mutation converted a 17b-resistant Env to one that was sensitive, with an IC_{50} of 17.2 μg/ml (Table 4; see Fig. S1 in the supplemental material). Despite not altering any known PG9/PG16 contact sites, the I423T/G589D mutant created a PG9- and PG16-resistant phenotype in a previously sensitive Env background. Similarly, the I423T/G589D mutant was >3-fold less sensitive to VRC01 than the bNAb-resistant clone.

Double amino acid substitutions in the MPER that confer neutralization sensitivity. We next examined the pair of Env proteins from subject 108069. We found that the wtR clone, 005, was neutralized only by the Z23 serum, at a titer of 1:196. In contrast, the wtS clone, 011, was neutralized by all three HIV-infected sera, at titers ranging from 1:120 to 1:581 (Table 6). Analysis of these envelopes was complicated by the fact that there were 20 amino acid differences between the wtS and wtR clones (Table 2). As can be seen in Fig. 1, the mutations occurred throughout the Env protein, in the C1, C2, V3, V4, C4, and V5 domains of gp120 and in the C34, MPER, and cytoplasmic domains of gp41. When the amino acid sequence differences were tested individually by site-directed mutagenesis (Table 6), no single point mutant could restore bNAb activity. However, a pair of mutations in the MPER (i.e., S668G and I675V) individually had small effects on neutralization sensitivity by the Z23 and Z1679 sera, but when combined, they restored neutralization sensitivity (Table 6). Studies with MAbs (Table 4) showed that wtR clone 005 was resistant to neutralization by b12, PG9, and CD4 IgG. Clone 005 was, however, moderately sensitive to PG16 and 4E10. When the S668G and I675V mutations were introduced into clone 005, we observed a 2-fold decrease in sensitivity to PG16, a 65-fold increase in neutralization sensitivity to 4E10, and a 10-fold increase in sensitivity to CD4 IgG. There was no change in sensitivity to the 17b or VRC01 MAb. Thus, this was another case where mutations in gp41 increased sensitivity to neutralization by CD4 IgG as well as by antibodies to the MPER.

DISCUSSION

In this paper, we compared the amino acid sequences of neutralization-sensitive and neutralization-resistant Envs isolated from virus quasispecies that evolved in each of five individuals infected with HIV-1. The viruses were all obtained at single time points close to the time of infection. Using site-directed mutagenesis, we were able to identify sequence polymorphisms responsible for differences in sensitivity/resistance to neutralization by bNAbs in three independent polyclonal HIV-positive sera. One significant finding from these studies was that all of the mutations we identified affected neutralization by all three of the HIV-infected sera. This result initially suggested that there might be overlap in the specificity of the bNAbs in all of these sera. However, subsequent studies showed that the increased sensitivity to neutralization was due to mutations in different parts of the molecule resulting in conformational changes that all conferred similar neutralization-sensitive phenotypes.

A second finding from these studies was that mutations in three discrete regions of gp120 (the V2 domain, the CD4 binding site, and gp41) can regulate neutralization sensitivity by causing conformational changes that simultaneously expose epitopes recognized by bNAbs in distinct regions of the Env protein. All of the mutations we identified occurred at positions that appear to have the potential to trigger biologically significant conformational changes in distal regions of the molecule. Amino acid positions with this capacity appear to be relatively rare, since we could identify pairs of neutralization-sensitive and -resistant viruses in only 12 of 57 subjects. Moreover, of the 91 single amino acid substitutions that we have evaluated to date, only 4 have this effect. While previous studies have identified the V2 domain as the global regulator of neutralization sensitivity (48, 53), our results suggest that additional regions of the Env protein (e.g., gp41 and the CD4 binding site) also have the capacity to determine neutralization sensitivity and resistance. Thus, we have identified three mutations in gp41 (Q653R, S668G/I675V, and L702P), three mutations in the V2 domain (D180N, D167G, and NI97H), and two mutations at or proximal to the CD4 binding site (Y384H and I423T) that all confer neutralization sensitivity. In one case, a mutation in

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gp41 (G589D) had little effect on neutralization itself but significantly enhanced neutralization when combined with the I423T mutation at the CD4 binding site (in subject 172297). Position 589 was previously reported to be in a region important for the interaction of gp120 and gp41 (2, 44).

Previous studies have reported polymorphisms in the C1 domain (25), the V2 domain (18, 40, 42, 48, 50, 57, 63), the α2 amphipathic helix in the C3 domain (50), the CD4 binding site (11, 12), and the C34 helix of gp41 (3, 19) that confer sensitivity and resistance to neutralizing monoclonal and polyclonal antibodies (12, 50). As described previously, we and others (3, 43) have suggested that mutations with increased neutralization sensitivity are more apparent in specimens from early infections, before high titers of neutralizing antibodies appear. Once these antibodies develop, the neutralization-sensitive variants are expected to be cleared from the plasma. It has also been reported that cooperation between the V1/V2 domain and the gp41 ectodomain is required for neutralization escape (51). Finally, it has been reported that the presence or absence of glycosylation sites can determine neutralization sensitivity or resistance (64). Since all of the mutations identified in this study increased sensitivity to CD4 IgG and the 4E10 MAb, it appears that the conformational changes induced by these mutations improved accessibility of antibodies to the CD4 binding site and to the MPER. These mutations appear to induce changes in quaternary interactions between protomers in the gp160 trimer and may alter the conformational equilibrium between the native and CD4-engaged conformations of the gp160 trimer (20, 35, 44, 61). Recent studies (29, 71) have suggested that the CD4-engaged conformation of gp120 is energetically more stable and results in a more open, antibody-accessible structure. These studies further suggested that one role of the V2 domain is to maintain this energetically less favorable structure that preserves conformational masking. The mutations we describe in this paper are consistent with the idea that there is a tendency for the Env protein to assume the more open CD4-engaged conformation, but the data suggest that other regions (e.g., sequences near the CD4 binding site and gp41) in addition to the V2 domain play an important role in maintaining the unliganded conformation.

In subject 172297, we observed a pair of mutations (Y384H and L702P) involving gp120 and gp41 that appeared to act independently to affect neutralization sensitivity/resistance. This double mutation is very likely to affect CD4 binding, since position 384 is known to be a contact for the potent CD4-blocking MAb b12 (72). We found that the Y384H mutation in combination with the L702P mutation resulted in a >40-fold difference in sensitivity. The identification of position 702 as a site that affects neutralizing antibodies was surprising. This position is located in the transmembrane domain of gp41 and is therefore not available for antibody binding. Previous studies have reported that mutations or truncation of the cytoplasmic tail can result in conformational changes that affect virus neutralization (13) and coreceptor usage (24).

Neutralization-sensitive clones from two subjects permitted independent identification of polymorphisms at positions 384 and 423, affecting the CD4 binding site. The locations of both amino acids relative to the CD4 binding loop are clearly visible (Fig. 2) in the gp120 crystal structure of Wu et al. (69). The amino acids are within a van der Waals binding radius of each other and potentially contribute to stabilizing tertiary contacts. Because the effect of each of these gp120 mutations is amplified by a mutation in gp41 (e.g., L702H or G589D), it is likely that the precise conformation of residues 384 and 423 in the CD4 binding site is affected by interactions with gp41. Hence, a single amino acid change in gp41 can apparently orient the virus spike in such a way as to alter the exposure of the CD4 binding site. This seems consistent with the observation that multiple conformational changes in gp41 appear to be triggered by CD4 binding (30).

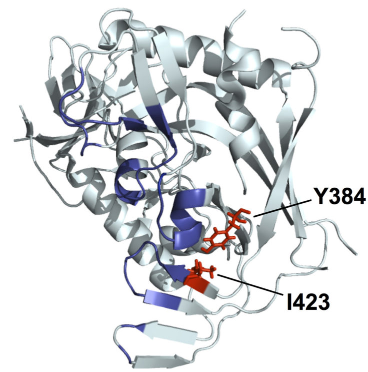

FIG 2 Locations of amino acid polymorphisms near the CD4 binding site that confer sensitivity/resistance to bNAbs in sera from HIV-infected individuals. The figure is based on the PDB 3SE8-1 structure of Wu et al. (69). The gp120 molecule is shown as a light cyan ribbon, with the locations of the CD4 binding loop and footprint shown in blue. The locations of the Y384H and I423T polymorphisms that confer neutralization sensitivity/resistance are shown in red.

In these studies, we identified two mutations in the V2 domain (D167G and N197H) that conferred neutralization sensitivity/resistance. Analysis of viruses from subject 172976 showed that the D167G mutation in the V2 domain determined neutralization sensitivity/resistance. This exact position was recently the subject of several investigations. D167 is a main chain contact residue for the broadly neutralizing PG9 MAb (38) and was recently shown (41) to occur at the exposed bend between the B and C strands of the 4-stranded β-sheet structure comprising the V1/V2 domain (Fig. 3). This position has also been implicated in the binding of multiple other neutralizing MAbs, including PG16, 2909, C108g, and 10/74b (16, 18, 23, 37, 41, 47, 63). In addition, this residue is important for the binding of a population of broadly neutralizing polyclonal antibodies in the CAP256 serum (40). The identification of the D167G polymorphism by swarm analysis confirms and extends previous results by showing that this site not only represents a target for neutralizing antibodies but also appears to be critically important for maintaining conformational masking. The D167 mutation serves to significantly alter the electrostatic features of the B-C junction by eliminating a negative charge that normally occurs between two positive charges (e.g., R166 and K168). It is tempting to speculate that the reason that antibody binding to D167 causes neutralization is because it disrupts an electrostatic interaction with another region of the molecule, possibly the V3 domain or the CD4 binding site, required to preserve the unliganded conformation and conformational masking.

The second V2 mutation identified in this study (N197H) occurs at a glycosylation site in the V2 stem, located in the D strand of the 4-stranded β-sheet structure. One can see (Fig. 3) that when a complex glycan is modeled at position N197 on V2, deletion of this large glycan structure will result in increased exposure of

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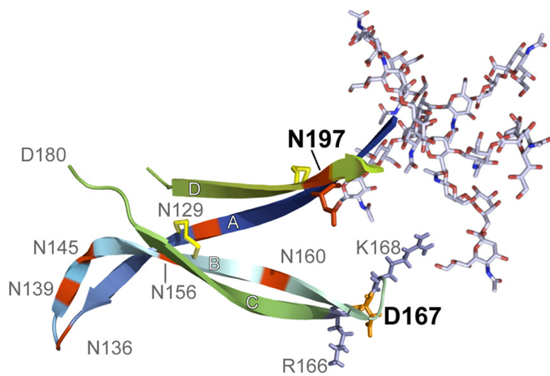

FIG 3 Locations of polymorphisms in the V1/V2 domain of gp120 that confer sensitivity and resistance to bNAbs in HIV-infected sera. The figure is modeled on the four-stranded β-sheet structure of the V1/V2 domain as described by McLellan et al. (38), with strands A to D indicated. The locations of the D167G mutation at the B-C junction and the N197H mutation at the C terminus of the D strand are indicated in bold. The predicted structure of the complex glycan attached to position N197 is provided based on GlyProt modeling (4). For reference, the locations of other predicted N-linked glycosylation sites in the V1/V2 domain are shown in red, with the specific positions of the asparagine (N) residue at each site indicated in gray text. However, the glycan structures attached to these sites were omitted for clarity. Disulfide bonds are indicated in yellow. The electrostatic surface of the B-C bend possesses distinct areas of charge, with the side chains of the basic arginine (R) and lysine (K) residues at positions 166 and 168 indicated in metallic blue. The location of the acidic aspartic acid (D) side chain at position 167 is shown in orange.

epitopes in the B-C junction, including positions 166 to 168, as well as at the C terminus of the D strand. Thus, this part of the V1/V2 domain appears far more accessible to antibody binding than the A-B junction, which appears to possess a high-density glycan shield. Position 197 has been noted by others as being critically important for neutralization sensitivity/resistance for several common laboratory strains of HIV, such as SF162 (36), 89.6 (33), and ADA (26, 27), as well as for CRF01_AE primary isolates (62). In clade B and C viruses, loss of the PNGS at position 197 also confers sensitivity to the b12 MAb, possibly by precluding glycan shielding of the CD4 binding site (68). Our data confirm and extend these observations related to position 197 by showing that polymorphism at this position occurs in unselected clinical isolates as well as lab-adapted viruses, and affects neutralization by multiple HIV-infected sera. These studies also suggest that bNAbs with similar specificities evolve in people from different parts of the world who are infected with different clades of virus, leading to the production of bNAbs that recognize the same epitopes and viruses that exhibit the same neutralization escape mutations. The Envs from subject 108069 were of interest because they exhibited the same I675V mutation in the MPER as that described previously (3). However, in the present study, the mutation occurred in a clade B virus, whereas this mutation was previously observed in a clade A virus. Thus, viruses from different clades appear to have evolved the same solution to create neutralization resistance.

Several additional conclusions can be drawn from the analysis of our data. First, we performed swarm analysis at only one time point for each subject. It would be particularly informative to carry out this type of analysis on longitudinal samples. Second, our results validate swarm analysis as an alternate strategy by which epitopes (e.g., position 167) recognized by bNAbs in polyclonal HIV-infected sera can be mapped without cloning B cells and isolating monoclonal antibodies. Third, our strategy provides an alternate approach to assess the breadth of neutralization induced by vaccines and monoclonal antibodies for the prevention and treatment of HIV-1 infection. Historically, the breadth of neutralization has been assessed with panels of single viruses (32, 55, 56) selected from a large number of different individuals. While this approach is useful for many purposes, it is limited in its ability to identify common polymorphisms responsible for neutralization sensitivity and resistance. Our results suggest that useful information related to the breadth of neutralization and common strategies for immune escape can be obtained by analysis of large numbers of viruses from the same individual. This approach has proven useful for characterizing resistance to antiviral drugs for HIV-1 (46) and may prove similarly useful for development of HIV-1 vaccines by highlighting common polymorphisms that confer neutralization sensitivity and resistance. Finally, the mutations we have identified will contribute to our understanding of the relationship between the structure and function of HIV-1 Env proteins by highlighting specific amino acid positions that appear to be important for maintaining quaternary interactions that affect neutralization sensitivity and resistance. These studies will add to our knowledge of the evolution of neutralization sensitivity and resistance and may aid in the design and selection of Env proteins to include in multivalent vaccine cocktails.

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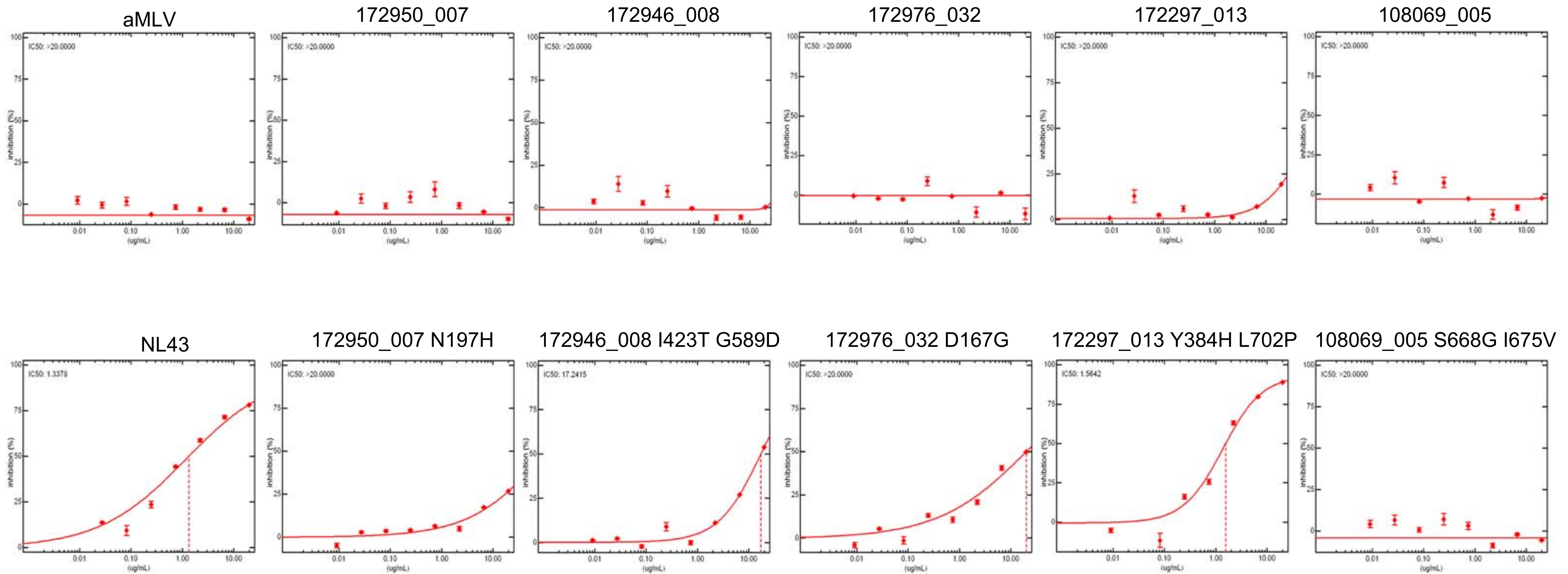
Supplemental Table S1. Neutralization of a multi-clade panel of viruses by the HIV-positive sera used in these studies.

Virus	Neutralization titer of indicated HIV-positive serum IC_{50} (1/dilution)		
	Z1679	N16	Z23
SF162	**4099**	**4720**	**22535**
1196	**70**	**142**	**413**
TRO	**215**	**63**	**297**
JRFL	**1011**	34	**514**
BG1168	**76**	21	**120**
QHO692	**36**	24	**137**
REJO	**64**	**62**	**515**
M-SC-B-006	**26**	<20	<100
APV-16	**97**	**90**	**572**
M-Chronic- B-013	**32**	23	**189**
PVO	**126**	28	**439**
M-A-002	<20	22	**181**
M-A-006	**52**	<20	**230**
M-C-003	**75**	23	<100
M-C-020	<20	<20	<100
M-D-006	**102**	<20	**238**
M-D-009	**135**	**135**	**476**
94UG103	<20	30	121
92BR020	**194**	**65**	**272**
93IN905	**301**	**91**	**326**
M-C-026	**36**	**55**	**277**
92TH021	**47**	**57**	**255**
JRCSF	**115**	**177**	**402**
JRCSF	**114**	**172**	**385**
NL43	**1021**	**769**	**3598**
NL43	**867**	**591**	**3172**
aMLV	<20	<20	<100

The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent significant neutralization titers that are at least three times greater than those observed against the negative control (aMLV).



Supplemental Figure S1.
Neutralization curves for monoclonal antibody 17b. A 10-point assay with 3-fold dilution series from 20µg/ml of antibody is shown for five pairs of neutralization-resistant and -sensitive envelopes in pseudovirus assay. Top panel, 17b activity against the five bNAb neutralization clones is shown. All clones were resistant to neutralization. Bottom panel, two out of five clones (172946_008_I423T/G589D and 172297_013_Y384H/L702P) were sensitive to b17 neutralization at IC50 <20µg/ml of antibody. Clones 172977_D167G and 172950_007_N197H showed a trend to neutralization by b17, but did not reach 50% inhibition at their highest antibody concentration (20 µg/mL). The laboratory isolate NL4-3 and aMLV represented positive and negative controls, respectively.