Continuous Viral Escape and Selection by Autologous Neutralizing Antibodies in Drug-Naïve Human Immunodeficiency Virus Controllers[▽]

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We assessed differences in the character and specificity of autologous neutralizing antibodies (ANAbs) against individual viral variants of the quasispecies in a cohort of drug-naïve subjects with long-term controlled human immunodeficiency virus type 1 (HIV-1) infection and moderate levels of broad heterologous neutralizing antibodies (HNAb). Functional plasma virus showed continuous *env* evolution despite a short time frame and low levels of viral replication. Neutralization-sensitive variants dominated in subjects with intermittent viral blips, while neutralization-resistant variants predominated in elite controllers. By sequence analysis of this panel of autologous variants with various sensitivities to neutralization, we identified more than 30 residues in envelope proteins (Env) associated with resistance or sensitivity to ANAbs. The appearance of new sensitive variants is consistent with a model of continuous selection and turnover. Strong ANAb responses directed against autologous Env variants are present in long-term chronically infected individuals, suggesting a role for these responses in contributing to the durable control of HIV replication.

Antibodies capable of neutralizing a subject's own virus, called autologous neutralizing antibodies (ANAbs), have been the subject of recent studies redefining the timing and character of this response. ANAbs develop early in essentially all seropositive subjects and increase in titer during the first few months and years of infection (15, 30). Previously published data were obtained using an assay that measures ANAbs against the complete quasispecies without an analysis of the individual envelope protein (Env) sequences to which these ANAb responses were directed (10). The contemporaneous virus pool was poorly neutralized, leading to an assumption that contemporaneous ANAbs are ineffective in controlling viremia. In chronic infection, ANAbs generally have been difficult to detect (3, 29, 31, 40), but there is ample evidence for selection by NAb and resulting virus *env* evolution in the host (12, 30, 38). The titers of ANAbs measured against clinical or autologous isolates cultured in peripheral blood mononuclear cells typically have been low in chronic infection (31, 40), while other studies indicated the presence of strong ANAbs (2). Although ANAbs may be ineffective in subjects with high virus loads due to the continuous generation of escape variants, their role in maintaining low viral loads in human immunodeficiency virus (HIV) controllers is not known.

NAbs that recognize heterologous isolates to which the subject has never been exposed, called heterologous NAbs (HNABs), are found later in infection, and not all subjects develop this broadening of the response (5). In studies that utilized easy-to-neutralize laboratory or primary viruses, titers of HNABs can be high (5, 6, 26, 29). Early work had shown that polyclonal HNABs in HIV-infected subjects are directed to conserved conformational determinants on gp120 (32), including the CD4-binding site (CD4bs) (22). Several human neutralizing monoclonal antibodies with broad activity also are directed to conserved conformational determinants on Env proteins, such as the CD4bs (4) and V3 (17). However, the mechanisms that lead to the development of broad HNABs are unknown. Their development likely is dependent upon the specific autologous Env proteins to which the subject is exposed, and these proteins are variants of the original infection in these subjects, except for cases of superinfection. Thus, we reasoned that a detailed analysis of the neutralization of individual autologous variants in subjects with broad responses and viral control could be informative.

The purpose of this study was to examine the autologous neutralizing responses against autologous viral variants in the plasma of HIV-positive subjects that were controlling infection for many years. These subjects have moderate HNABs against the quasispecies of other subjects (27). We compared longitudinal samples from five chronically infected, antiretroviral treatment-naïve adults late in infection. Despite the short time frame between the sample time points, the amount of *env* variation was surprisingly high, indicating continuous viral evolution in controllers; contemporaneous ANAbs were present and maintained in all except one elite controller. We cloned individual *env* gp160 plasma variants and analyzed sequence changes related to the autologous neutralization sensitivity or resistance. We systematically examined the ANAb response

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[▽] Published ahead of print on 5 November 2008.

TABLE 1. Subject characteristics^a

Subject	Yr of diagnosis	Category ^b	Plasma sample date ^c (mo/day/yr)	Time point	Virus load (RNA copies/ml plasma)	CD4 count (absolute)
EC1	1990	Elite controller	9/26/05	T1	74 (<75 cutoff)	843
			4/13/06	T2	NA ^d	NA
			7/12/06	T3	NA	NA
EC2	1985	Elite controller	8/2/05	T1	49	1,005
			11/14/06	T2	49	1,024
VC1	1985	Viremic controller, now with breakthrough viremia	3/14/05	T1	27,500	630
			11/15/05	T2	15,900	580
			3/14/06	T3	13,300	612
VC2	1989	Viremic controller	3/23/04	T1	5,020	812
			2/3/05	T2	1,430	858
			9/10/05	T3	3,290	671
VC3	1989	Viremic controller, now with breakthrough viremia	5/18/05	T1	4,243	943
			6/22/05	T2	9,000	930
			10/7/05	T3	695	959
			4/7/06		1,078	950
VC4	1989	Viremic controller	3/30/07		1,875	764
			5/3/04	T1	354	702
			8/16/04	T2	110	557
			3/14/05	T3	74 (<75 cutoff)	654
VC5	1991	Viremic controller	12/28/05	T4	74 (<75 cutoff)	655
			1/21/03	T1	1,140	405
			4/14/03	T2	181	NA
			7/21/03	T3	1,130	NA
VC6	1998	Viremic controller	11/20/03		576	708
			6/3/04	T1	NA	439
			9/9/04	T2	845	735
			12/23/04	T3	913	650
VC7	1990	Viremic controller	8/9/05	T4	399 (<400 cutoff)	478
			2/26/04	T1	1,160	619
			4/15/05	T2	3,250	NA
VC8	1992	Viremic controller	4/10/05	T1	1,180	628
			7/28/05	T2	7,280	330
			11/9/05	T3	1,740	511
VC9	1992	Viremic controller	6/11/03	T1	2,533	835
			10/31/03	T2	1,052	847
			3/14/04	T3	298	340
VC10	1991	Viremic controller, now with breakthrough viremia	9/23/04	T1	1,780	625
			12/21/04	T2	10,200	390
			4/6/05		505	902
VC11	1998	Viremic controller	4/13/06	T3	8,150	496
			11/3/00	T1	15,000	NA
			10/23/01	T2	667	375
			10/17/03	T3	1,670	NA

^a Antiretroviral treatment status is naïve for all subjects.

^b Subject classifications are according to plasma virus load as follows: elite controllers, <50 copies/ml; aviremic subjects, 2,000 copies/ml or less but more than 50 copies/ml; aviremic with breakthrough, the recent loss of viremic control with sustained spikes of >10⁴ copies/ml.

^c Dates of longitudinal plasma samples analyzed for autologous and heterologous NAb and gp160 sequencing.

^d NA, not available.

directed to individual variants using contemporaneous and noncontemporaneous plasma samples and observed patterns that have not been previously reported. Mutations that were significantly associated with sensitivity or resistance to ANAbs were found on parts of the envelope that are exposed and thus may be accessible to antibodies, consistently with a role in escape and containment by NAbs.

MATERIALS AND METHODS

Study subjects. Blood samples from HIV type 1 (HIV-1)-infected individuals were obtained from outpatient clinics at the Massachusetts General Hospital, affiliated Boston hospitals, and providers throughout the United States. The respective institutional review boards approved the study, and all subjects gave

written informed consent. Thirteen antiretroviral treatment-naïve subjects with long-term chronic HIV infection who had been diagnosed 15 to 22 years earlier (clade B from the United States) were analyzed for heterologous NAbs. We selected persons (with levels of CD4⁺ T cells of >400/mm³) of two subsets: (i) elite controllers for whom the level of HIV RNA without antiretroviral therapy was below the level of detection for the respective assay (e.g., <75 copies measured by branched DNA assay or <50 copies by ultrasensitive PCR) and (ii) viremic controllers for whom HIV RNA levels without antiretroviral therapy were below 2,000 copies/ml. Some subjects had episodes of viral breakthrough with viral blips of >2,000 copies/ml plasma, followed by subsequent decreases in RNA levels (Table 1).

Env gp160 cloning and autologous pseudovirus construction. To determine the neutralization susceptibility of viral variants from HIV-1 controllers, single-cycle-competent pseudoviruses were generated. Full-length *env* gp160 was amplified from plasma of viremic controllers VC1, VC2, and VC3 via two-step

reverse transcriptase PCR. First, cell-free viral RNAs from plasma samples were isolated using Qiagen viral RNA mini kits according to the manufacturer's instructions. Two-step reverse transcription-PCR (RT-PCR) of plasma viral RNA was performed using a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) to generate cDNA. In order to increase the number of copies of viral cDNA for ease of amplification in the downstream steps, maximal RNA concentrations of up to 1 µg and random hexamers were used to prime the RT step. Multiple replicates (~10 to 40) for a subject sample were included to increase the chances of obtaining a positive result. Nested PCR was performed on 5 to 10 µl of cDNA from each replicate RT reaction with Expand Hi-Fidelity *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN). Specific primers matched to the database HxB2 sequence (accession number AF033819) were used. gp160 first-round primers were EO (TAGAGCCCTGGAAGCATCCAG GAAGTCAGCCTA) and EO1 (TCCAGTCCCCCTTTTCTTTTAAAAA). First- and second-round cycling conditions for gp160 PCR were the following: denaturation at 95°C for 5 min; 10 cycles of 94°C for 40 s, 50°C for 30 s, and 68°C for 3 min 15 s; 25 cycles of 94°C for 40 s, 60°C for 30 s, and 68°C for 3 min 15 s; and a final elongation at 68°C for 10 min. Second-round primers for gp160 amplification were NheI gp160 P3 F, which inserts an NheI site at the 5' end of gp120 to fuse with the t-PA signal in the expression vector pEMC* (GCGGCG GCGGCTAGCGTAGAAAAATTGTGGGTAC), and P3 gp160 R ClaI, which inserts a ClaI site at the 3' end of gp41 (GCCGCGCCATCGATTATAGC AAAGCCCTTTC). Positive controls for the RT step included RNA containing 10 and 100 copies of HIV-1 RNA, and the PCR step included genomic DNA containing 1, 10, and 100 copies of HIV-1 DNA. Negative controls included nuclease-free water and HIV-negative RNA and DNA. PCR products from multiple replicate reactions were purified separately using the Promega Wizard SV gel and PCR clean-up system (Promega Corp., Madison, WI).

For elite controllers EC1 and EC2, 16 and 30 ml of patient plasma, respectively, was used to pellet the virus. In brief, the large volume of plasma was spun down for 10 min at 1,500 rpm to remove cell debris. Virus was concentrated by ultracentrifugation at 124,513 relative centrifugal force units for 2 h using an SW32 Ti rotor (Beckman Coulter, Fullerton, CA). Supernatant was removed, leaving 140 µl of the sample, and viral RNA was extracted by a Qiagen viral RNA mini kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). In this process, after the column was washed with buffer AW1, DNase treatment was performed on the column using a Qiagen RNase-free DNase set, and then the buffer AW1 wash was repeated. Viral RNA was eluted in 80 µl of DNase-RNase-free water and stored at -80°C until use.

First-round RT-PCR was performed using a Superscript III one-step RT-PCR system with Hi-Fidelity platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The reaction mixture (50 µl) was composed of 10 µl of RNA, 25 µl of 2× reaction mix, 400 nM of forward primer FB6 (GCATTCCTACAATCCCCA AAG, 4645 to 4666; numbered with respect to the reference strain HXB2 sequence in the Los Alamos HIV database) and reverse primer FB12 (GCAC TCAAGGCCAAGCTTTATTGAGG, 9629 to 9603), 1 µl of enzyme mix, and water. RT-PCR cycling conditions were 1 cycle of 30 min at 55°C and 2 min at 94°C; 40 cycles of 15 s at 94°C, 30 s at 60°C, and 5 min at 68°C; and a final elongation at 68°C for 5 min. Second-round primers and cycling conditions were the same as those described above for the viremic controllers.

Inserts (2.5 kb gp160) were cloned into an expression vector, pEMC*, following double restriction digestion with NheI and ClaI and ligation with a Roche rapid DNA ligation kit (Roche Diagnostics, Indianapolis, IN). MAX Efficiency Sbl2-competent cells (Invitrogen) were transformed with 10 to 20 ng of the ligation reaction mixture according to the manufacturer's instructions and grown at 30°C for 24 h. We obtained 5 to 10 functional pseudoviruses by screening 100 to 200 transformants on average. Transformants were plasmid purified with the Qiagen miniprep kit as previously described (18). 293T cells were transfected with 1 mg/ml of polyethylenimine (PEI; Polysciences, Inc., Warrington, PA). 293T cells (4×10^5 per well) were plated to 50% confluence in 6-well plates 24 h prior to the transfection; 1 µg total DNA with a 20:1 backbone/Env ratio was prepared with 10.5 µg PEI in a Dulbecco's modified Eagle's medium (DMEM) mixture. Virus was harvested 48 to 72 h later, spun at 2,000 rpm for 10 min, and stored at -80°C until use. The titers of the pseudoviruses were determined on TZM-bl cells (37), which were provided by N. Landau via the NIH AIDS Reagent Repository, to obtain 200 50% tissue culture infectious doses.

Neutralization assay. Only envelopes that were positive for virus infection in the in vitro virus entry TZM assay were sequenced and used in the neutralization assay. Briefly, 200 50% tissue culture infectious doses of virus were added to twofold serial dilutions of sera in the presence of 7.5 µg/ml DEAE-dextran for 1 h at 37°C. Each well received 100 µl of TZM-bl cells resuspended in DMEM containing 10% fetal calf serum, 1% L-glutamine, 1% penicillin, 1% streptomycin at 1×10^5 cells/ml. Forty-eight hours later, cells were lysed for 2 min directly in

the neutralization plate using 100 µl of Bright-Glo luciferase assay substrate (Promega, Madison, WI) and immediately analyzed for luciferase activity on a luminometer. The reciprocal dilution of serum necessary to achieve 50% neutralization is reported. Neutralization values greater than three times the background neutralization value for a negative control human serum (Sigma, St. Louis, MO) were considered significant. The lowest dilution of the negative control human serum tested (1:50) never reached 50% neutralization; therefore, titers ≥ 60 were considered positive for neutralization. Positive controls included pooled human sera or purified immunoglobulin G from HIV-1-infected subjects previously described as having broad NAb responses against clade B isolates (J. Mascola and D. Montefiori, unpublished data). All values were calculated with respect to virus only using the following formula: [(value for virus only - value cells only) - (value for serum - value cells only)]/(value for virus only minus value for cells only). Assays for heterologous neutralization were performed as described above against 10 HIV-1 clade B-pseudotyped virus isolates from the recommended panel (21). The heterologous panel viruses used are listed below for the maximum-likelihood tree.

Sequence analyses. The 5 to 10 functional *env* genes used in the pseudovirus construction and neutralization assays were sequenced in both directions using vector primers pEMCF2 (GTGTGCTGCTGCTGTGTGG) and pEMCR2 (GAT CATTACTTATCTAGGTCGACTG) and *env* primers ED5P3mod (ATGGGAT CAAAGTCTAGAGCCATGTG), KK1 (GCACAGTACAATTGTACACATGG AA), 218 (ATCATACACTTTAGAAATCGC), ED3 (TTACAGTAGAAAAA TTCCCC), ED8FOR (TGAGGGACAATTGGAGAAGTG), and ED12FOR (CTTGGGTTCTTGGGAGCAGCAGGAAGCACT) (11) using Prism dye terminator kits (ABI, Foster City, CA) on an Applied Biosystems 3730XL genetic analyzer. Plasma viral *env* nucleotide sequences were edited and assembled with Sequencher4.5 (Gene Codes Corporation, Ann Arbor, MI) and aligned using CLUSTALX (8, 35). Consensus B (ConB) (consensus and ancestral alignments tool; Los Alamos HIV sequence database; www.hiv.lanl.gov) (16) and HxB2 (accession number AF033819) were used as reference strains for sequence analyses. Percent diversity and sequence heterogeneity at a particular time point in a sample from a subject were calculated using MEGA3.0 (20) using the Kimura two-parameter model with pairwise deletions (transition-to-transversion ratio [k] of 2). The divergence of each clone sequence from that of ConB was measured with MEGA3.0 using the Kimura two-parameter model with pairwise deletions ($\kappa = 2$). The average percent diversity per subject was calculated by averaging the percent diversity of sequences at each time point.

Maximum-likelihood analyses. Codon-aligned nucleotide sequences trimmed in length to the same starting and ending nucleotides were analyzed by maximum likelihood using PAUP*4.0 (D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA) (34). Trees were rooted on the ConB sequence. Maximum-likelihood trees ($\kappa = 2$) were created using the HKY85 model with a subtree-pruning regrafting branch-swapping algorithm. The starting tree was obtained by neighbor joining, and the starting branch lengths were obtained using the Rogers-Swofford approximation method. HXB2 (accession number AF033819) and clade B sequences belonging to the standard reference panel for testing neutralization (QH0692, PVO, TRO, AC10.0, WITO, REJO, RHPA, THRO, CAAN, and SC422661; accession numbers AY835439, AY835444, AY835445, AY835446, AY835451, AY835449, AY835447, AY835448, AY835452, and AY835441, respectively) were included as reference strains on the phylogenetic tree. Outlier sequences included clade C TV001c8.5 and TV001c8.2, accession numbers AF391231 and AF391230, respectively, clade D FIN93167 and FIN93178, accession numbers AF219271.1 and AF219272.1, respectively, and clade G NG1939 and NG1937, accession numbers AF069935.1 and AF069937.1, respectively.

Analysis of glycosylation and amino acid mutation. AminoTrack was used to identify potential N-linked glycosylation (PNG) sites and differences in amino acid identity within each Env sequence. Protein sequences were obtained (Sequencher4.5) and aligned (CLUSTALX) using HIV-HXB2 (accession no. K03455) for reference to standardized amino acid position numbering. This web-based software (<http://apps.sbri.org/AminoTrack/>) (23) provides multiple spreadsheet outputs that denote the amino acids altered between each sequence in a protein alignment and positions at which a PNG site is present. Sequences motifs of NxS/Tx, where x is any amino acid except for proline, are recognized by the program as a PNG site. PNG site positions were noted, and the number of PNG sites per autologous virus variant were tallied. These spreadsheets were imported into the statistics program SPSS (SPSS Inc., Chicago, IL) for the statistical analysis of significant amino acid and PNG site differences correlated with the neutralization sensitivity of an Env sequence.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 3.0, SPSS, R, and QVALUE software packages. The nonparametric Mann-Whitney U test was used to compare the continuous outcomes of two

TABLE 2. Assessment of heterologous neutralization

Subject plasma and time point ^a	HNAb titer for subtype B HIV-1 <i>env</i> clone ^b :										No. neutral-ized (<i>n</i> = 10 ^c)
	SC 422661	TRO	REJO	CAAN	PVO	QH 0692	RHPA	AC10	THRO	WITO	
VC4 T1	200	170	70	150	1,600	150	80	150	150	50	9
VC4 T4	340	250	138	111	371	156	73	324	100	56	9
VC2 T1	150	160	200	80	3,200	100	100	100	—	50	8
VC2 T3	125	251	51	138	93	53	84	131	—	84	7
VC5 T1	400	180	200	—	200	100	170	—	400	—	7
VC5 T3	340	404	176	—	122	390	402	—	84	64	8
EC1 T1	174	288	145	—	—	348	126	62	ND	ND	6
EC1 T3	224	398	174	61	69	400	234	112	ND	ND	8
VC1 T1	200	80	250	200	400	—	—	—	100	100	7
VC1 T3	164	98	98	—	131	181	340	—	53	—	6
VC6 T1	— ^f	50	80	>3,200	200	—	—	—	—	200	4
VC6 T4	78	120	98	98	80	55	73	—	54	582	7
VC7 T1	224	80	71	50	67	168	389	—	—	—	6
VC3 T3	304	78	93	—	—	—	60	—	—	73	5
VC8 T3	73	—	—	84	—	51	—	71	71	—	4
EC2 T1	68	90	—	50	50	83	—	68	ND	ND	4
EC2 T2	102	170	50	71	—	132	50	—	ND	ND	4
VC9 T1	—	80	80	150	3,200	—	—	—	—	—	4
VC9 T3	50	84	82	311	—	—	—	—	—	—	3
VC10 T3	84	—	—	—	—	87	—	—	84	—	3
VC11 T1	50	—	50	800	—	—	—	—	—	—	1
VC11 T3	—	—	—	—	—	54	50	—	—	—	0
No. neutralized out of 22 plasmas ^d	17	16	14	14	13	12	11	9	8	5	
Controls											
Negative control ^e	—	—	—	—	—	—	—	—	—	—	0
Panel B serum pool ^g	509	>3,200	433	64	313	220	291	260	196	1,038	10

^a Plasma was from the HIV controller cohort.^b Standard reference panel of subtype B HIV-1 *Env* clones for the assessment of HNAb (21). Values are the 50% neutralization titers (50% inhibitory concentrations). ND, not determined.^c The number of heterologous viruses neutralized (titer $\geq$ 60).^d The number of subject plasmas positive for panel B virus neutralization (titer $\geq$ 60).^e HIV-negative human sera (Sigma, St. Louis, MO).^f The dashes denote titers of <50, the lowest plasma dilution tested, at the limit of detection.^g Pool of sera from HIV-1 clade B-infected subjects with broadly cross-reactive NAb against panel B viruses (D. Montefiori, unpublished results).

groups. Nonparametric two-tailed Spearman correlation coefficients were used to estimate correlation coefficients. A conservative false discovery rate of 0.013 was used to determine which mutations conferring changes to PNG sites and amino acids were significantly associated with susceptibility or resistance to ANAb (33).

Structural mapping of *Env* mutations and PNG sites. The amino acid and PNG positions correlated with *Env* resistance or susceptibility to autologous neutralization were mapped onto the three-dimensional HIV-1 gp120 monomer. The *Env* structure previously was generated by modeling the V1, V2, and V3 loops onto the YU2 core X-ray structure (Protein Database code 1RZK) as described in detail by Blay et al. (1). In the present analysis, PyMOL (PyMOL Molecular Graphics System, v0.99; DeLano Scientific, Palo Alto, CA) was used to show the locations of the amino acids and PNG sites in the structure.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited in GenBank under accession numbers FJ147091 to FJ147150.

RESULTS

HNAb responses against the HIV-1 clade B panel. The goal of this study was to investigate the relationship between ANAbs and the *Env* proteins to which responses are targeted in subjects with HNABs. HNAb responses in viremic (*n* = 17) and elite (*n* = 17) controller subjects in this study (Table 1) previously were tested in the Monogram assay by analyzing the ability of plasma to neutralize the quasiespecies of other subjects within the cohort (28). HNABs capable of neutralizing the

quasiespecies, when present, had more frequent responses and higher titers in the viremic group than in the elite controller group (*P* < 0.0005). Subject plasma samples were obtained between 2003 and 2006. We tested plasma samples from two time points, 4 to 18 months apart, in a selected subset of 11 viremic controllers and 2 elite controllers for HNAb titers (50% inhibitory concentrations) against a panel of clade B Tier 2 concurrent isolates (21). Overall, HNAb responses were low but reproducible, in the range of the responses of the positive control serum pool; the breadth of responses was defined by the number of heterologous isolates neutralized (Table 2). The breadth of HNAb responses varied by subject, with no significant difference in the magnitude of HNAb titers between the viremic subjects and the elite controllers (*P* = 0.6). There was no correlation between the percentage of heterologous viruses neutralized in this panel and the plasma virus load (*P* = 0.418; correlation coefficient ρ = -0.26). To address the question of the stability of the HNAb response, we compared the breadth at two different time points 4 to 18 months apart (Table 1). We observed the neutralization of 0 to 10 panel viruses, and HNABs varied in specificity and magnitude of response at these relatively close time points for each subject. Although the number of viruses neutralized was the same or

greater at the later time point, we did not observe consistent increases in titers against individual heterologous pseudoviruses over time. Titers increased against certain heterologous isolates and decreased or remained the same against other isolates in the subjects studied (Table 2). Thus, we predicted that the *env* genes in these subjects continued to mutate in regions affecting neutralization, even in the face of apparently low viremia.

We cloned and characterized full-length plasma gp160 *env* from several time points for five HIV controllers with differing degrees of HNAbs responses. We chose viremic subjects VC1, VC2, and VC3 and elite controllers EC1 and EC2, which are representative of broader and narrower HNAbs responses and for which longitudinal plasma samples were available. Subject *env* sequences were analyzed for phylogenetic relatedness to each other and to those from the clade B Tier 2 panel (Fig. 1). *env* genes were approximately 10% divergent from the clade B consensus sequence, as would be predicted given the length of the infection of these subjects (>10 years). Sequences from each subject clustered separately, confirming that all subject viruses were phylogenetically unlinked. With the exception of subject VC3, all sequences obtained at different time points also clustered separately. The distance between subject sequences and the panel B viruses ranged from 12.1 to 16.4%. We analyzed the percent distance between subject sequences and heterologous viruses and found no significant association of HNABs with phylogenetic distance. Neither early nor late HNAbs titers (for early HNAbs, $P = 0.631$ and $\rho = -0.073$; for late HNAbs, $P = 0.417$ and $\rho = -0.14$) nor the percentage of panel viruses neutralized ($P = 0.6833$; $\rho = -0.3$) was significantly associated. The HNAbs response also did not correlate with the average percentage of diversity of *env* genes ($P = 0.133$; $\rho = 0.8$).

ANAB responses against cloned plasma *env* variants. Functional cloned Env variants were pseudotyped from two to three time points each and were tested against autologous plasma (Table 3). The neutralization susceptibility of individual Env variants varied considerably; most were sensitive to the positive control, which was a pool of sera from HIV-1 clade B-infected patients with broadly cross-reactive NABs against panel B viruses. In some cases the neutralization of a variant increased over time within the subject, such that titers from time point 1 (T1) to time point 3 (T3) or time point 2 (T2) to T3 and the titers against viruses at earlier time points were greater than those against contemporaneous or later virus (VC1, T2, clone 1 [designated VC1 T2c1] and VC2 T1c2). Other variants had lower titers at T2 or T3 than at T1 (VC2 T2c3), suggesting a waning of NAB responses to these variants. These patterns previously were reported against individual variants and the quasiespecies as a whole in acute and early infection (30, 38). More frequently, we observed a pattern of ANABs that has not

been reported previously. For all time points analyzed, the majority of variants in all subjects were (i) sensitive at early and late NAB time points but more resistant at the intermediate plasma NAB time points (e.g., VC1 T3c6) or (ii) were consistently neutralized at the same level by ANAB for all time points (e.g., VC3 T2c3). Variants were categorized as (i) resistant to ANAB if the ANAB titers were <60, (ii) sensitive to ANAB if titers ranged between 60 and 700, and (iii) highly sensitive when ANAB titers were >700.

In subject VC1, the neutralization of variants derived from all three time points was similar, with a wide range of sensitivities to ANAB (Fig. 1, Table 3). At each time point, one or two highly neutralization-sensitive variants were detected along with a majority of variants with 10- to > 70-fold greater neutralization resistance. Unlike VC1, VC2 and VC3 variants increased in resistance over time, with the VC2 neutralization of T3 variants being significantly lower than those of T1 and T2 variants ($P < 0.0001$ and $P = 0.02$), and those of VC3 T3 variants were significantly lower than those of T2 variants ($P = 0.0293$) (Table 3). This is consistent with observations from acute infection (38). In the case of subject VC3, though each sequence is unique, most of the sequences showed less diversity, except for two clones from the first time point, T1c1 and T1c2 (Fig. 1). These clones form a separate cluster on a separate branch within the VC3 sequences, yet they are related to the other VC3 sequences through a common node connecting directly only to T2 and T3 sequences. As T1c1 and T1c2 pseudoviruses are the only variants resistant to the autologous neutralization that we detected, these Env variants may be neutralization escape variants that persist and evolve into T2 and T3 viruses in VC3. The majority of T3 viruses phylogenetically linked to the T1 sequences are similarly neutralization resistant, with the exception of T3c2. The remaining T1 sequences at the bottom of the tree are closely related to some T2 sequences. These T1 and T2 variants all are similarly sensitive to ANAB. For subject VC2, T2 variants c3 and c4 cluster separately from the other T2 variants and are more closely related to the T3 sequences than the main T2 cluster. These T2 variants are highly sensitive to ANAB, unlike the other two T2 variants, which show 10- to 50-fold higher levels of resistance.

Similarly to the viremic controllers, elite controller EC1 had variable ANAB responses against Env variants of diverse sequences, with half of the variants resistant and half moderately sensitive to ANAB (Table 3). In contrast, all EC2 variants had identically resistant ANAB titers (Table 3), which represents a lack of autologous responses. The sequences show little variation and have the lowest overall diversity among all subject sequences (Fig. 1).

NAB responses typically are low in subjects with tightly controlled viremia, most likely due to low antigenic thresholds resulting in minimal *env* evolution from the prolonged control

FIG. 1. Phylogenetic analyses of full-length functional *env* sequences in viremic controllers (VC1, VC2, and VC3) and elite controllers (EC1 and EC2). The maximum-likelihood tree is rooted on the clade B consensus gp160 (ConB) from the Los Alamos HIV sequence database. Also included on the phylogenetic tree are HxB2, representative sequences from clades C, D, and G as outlier sequences, and 10 gp160 sequences of the clade B panel used in the heterologous neutralization assays. Accession numbers for these sequences are listed in Materials and Methods. The susceptibility of patient Env to autologous neutralization is illustrated in color (red, neutralization resistant to ANAB, with titers of <60; green, sensitive to ANAB, with titers ranging between 60 and 700; and blue, highly sensitive Env proteins, with ANAB titers of >700). Individual sequences are named for the time point (T1, T2, and T3) and clone number (c1, c2, etc.).

TABLE 3. Autologous neutralization

Variant ^a	Plasma NAb titer at ^b :			Positive control ^c	Negative control ^d
	T1	T2	T3		
VC1					
T1c1	200	72	152	132	— ^e
T1c2	178	138	178	80	—
T1c3	126	100	126	65	—
T1c4	682	682	815	872	—
T2c1	80	80	330	1,260	—
T2c2	1,600	1,600	>3,200	2,400	—
T2c3	250	100	307	670	—
T2c4	200	180	230	400	—
T2c5	340	78	301	262	—
T3c1	99	70	222	>800	—
T3c2	>3,200	>3,200	>3,200	1,250	—
T3c3	—	100	160	1,440	—
T3c4	305	400	520	1,610	—
T3c5	>1,600	1,600	>1,600	>3,200	—
T3c6	230	—	100	660	—
VC2					
T1c1	128	100	150	68	—
T1c2	200	262	353	—	—
T1c3	133	116	207	100	—
T1c4	150	130	252	150	—
T1c5	113	93	169	219	—
T2c1	60	—	65	160	—
T2c2	70	65	90	200	—
T2c3	900	710	450	450	—
T2c4	>3,200	1,600	1,200	1,200	—
T2c5	—	—	—	—	—
T3c1	75	—	75	—	—
T3c2	80	—	80	—	—
T3c3	—	—	60	—	—
T3c4	—	—	—	—	—
T3c5	—	—	—	—	—
VC3					
T1c1	50	—	50	125	—
T1c2	—	—	—	2,500	—
T1c3	290	310	266	403	—
T1c4	235	250	285	455	—
T1c5	280	400	330	417	—
T1c6	250	270	230	500	—
T1c7	515	515	370	305	—
T1c8	400	450	475	248	—
T2c1	400	400	400	521	—
T2c2	125	139	200	—	—
T2c3	267	252	252	—	—
T2c4	58	86	102	—	—
T2c5	75	81	71	—	—
T2c6	267	267	237	—	—
T3c1	—	—	50	1,667	—
T3c2	315	315	300	313	—
T3c3	—	—	—	2,500	—
T3c4	—	—	80	807	—
EC1					
T2c1	400	280	430	192	— ^e
T2c2	—	—	—	417	—
T2c3	52	60	140	291	—
T2c4	110	110	90	212	—
T2c5	52	52	—	347	—
T2c6	—	—	—	781	—
EC2					
T1c1	—	—	—	362	— ^e
T1c2	50	—	—	274	—
T1c3	—	—	—	521	—
T1c4	—	—	—	521	—
T1c5	—	—	—	417	—
T1c6	—	—	—	500	—

^a Unique, missense mutation-free, full-length *env* sequences (gp160) from HIV-1 controllers were cloned, sequenced, and used for pseudovirus production.

^b The 50% neutralization titers (50% inhibitory concentrations) are shown. Boldface numbers indicate neutralization of individual variants using contemporaneous autologous plasma.

^c For the VC1 variants, the pool of sera from HIV-1 clade B-infected patients with broadly cross-reactive NAb against panel B viruses served as the positive control (D. Montefiori, unpublished). For the VC2, VC3, EC1, and EC2 variants, immunoglobulin (measured in micrograms/milliliter) purified from sera of HIV-1 clade B-infected patients with broadly cross-reactive NAb against clade B panel viruses served as the positive control (D. Montefiori, unpublished).

^d HIV-negative human sera served as negative controls (Sigma, St. Louis, MO).

^e Dashes denote titers of <50, the lowest plasma dilution tested, at the limit of detection.

FIG. 2. ANAb responses in viremic patients (VC1, VC2, and VC3) and elite controllers (EC1 and EC2). The relationship between plasma virus load and the level of autologous NAb responses was determined. The 50% inhibitory neutralization titers of each plasma sample against each variant from viremic and elite controllers were analyzed (using the two-tailed Mann-Whitney U test and 95% confidence intervals).

of virus replication. This is consistent with the observation that *env* sequences that form an intrasubject phylogenetic cluster are similarly neutralized by ANAb. In comparing the various subjects there was a range of diversity, with subject VC2 exhibiting the highest diversity at 1.5% and EC2 the lowest at 0.2%. Subjects with greater *env* diversity had a greater range of ANAb titers against the variants (Fig. 1, Table 3). For example, within a single time point the variation in 50% ANAb titers among clones ranged from 80 to 1,600 or 330 to more than 3,200 in VC1 T2 variants c1 and c2. Similar ranges of variation were observed in VC3, VC2, and EC1 variants. EC2 showed the least diversity and no variability between ANAb titers.

Both ANAb and HNAAb data in this study were obtained using pseudoviruses generated by the same methods and tested in the same cell line reporter assay, thus allowing us to compare the relative strength of these two types of responses. The magnitude of ANAb responses in all subjects combined (Table 3) was greater than the magnitude of their heterologous responses (Table 2) against the clade B panel of pseudoviruses ($P = 0.0002$). We asked whether there was a relationship between plasma virus load as a measure of virus replication and the overall level of ANAb titers. ANAb titers were significantly greater in viremic subjects than in elite controllers ($P < 0.0001$). The subjects with breakthrough viremia, VC1 and VC3, have the highest levels of ANAb responses against their variants, followed by VC2, which was viremic without having breakthrough viremia, and elite controllers EC1 and EC2, with <50 copies/ml. Numerical titers did not correlate with virus load, as VC2 plasma neutralized some autologous variants with titers as high as those observed in VC1 and VC3 plasma at the times of sampling. In this subject set, we observed a strong positive correlation between plasma virus load (in copies per milliliter) and the level of autologous neutralization (Table 1,

TABLE 4. Mutations that modulate neutralization phenotype according to glycan positions

ANAb phenotype ^a	PNG position ^b	Env location	Correlation ^c	P value
Sensitive	145	V1-V2	0.417	0.001
	148	V1-V2	0.345	0.007
	148f	V1-V2	0.421	0.001
	234	C2	0.478	<0.001
	295	C2	0.460	<0.001
	397a	V4	0.339	0.008
Resistant	460a	V5	0.345	0.007
	49	C1	-0.501	<0.001
	135	V1-V2	-0.460	<0.001
	142	V1-V2	-0.472	<0.001
	407	V4	-0.463	<0.001
	411	V4	-0.339	0.008

^a The presence of a PNG at these positions is correlated with sensitivity or resistance to ANAb.

^b Numbered with respect to reference strain HXB2.

^c Determined by two-tailed Spearman rank correlation and 95% confidence intervals.

Fig. 2). Virus load was associated with the percentage of variants neutralized at all time points ($P = 0.0167$; $\rho = 0.9747$) and the percentage of positive titers (i.e., those that are above the cutoff) for ANAb ($P = 0.0167$, $\rho = 1.0$).

Modeling of amino acids potentially affecting neutralization. Mutations conferring changes to potential PNG sites and amino acids significantly associated with either susceptibility or resistance to ANAb (Tables 4 and 5) were detected in Env variants ($n = 61$) from all subjects. These residues were mapped onto the YU-2 model of HIV-1 gp120 monomer to determine their location on the three-dimensional protein structure derived previously (1). The majority of these residues mapped either to the outer domain or to the variable loops, with the exception of six positions in C1 (49 and 89) and C2 (227, 234, and 236), which map to the inner domain, and position 219 in C2 at the junction of the inner and outer domains (Fig. 3).

We observed unique differences in the locations of 11 PNG sites and 22 amino acid substitutions that correlated with either sensitivity or resistance to ANAb (Table 4). For example, two mutations in the V3 loop crown at positions 314 (the second G in the GPGR motif) and 318 were correlated with increased sensitivity to ANAb but not with resistance. In contrast, a distal mutation in V3 at position 305 was correlated with a resistance phenotype but was not present significantly in sensitive Env sequences. Other regions of note include the C3 $\alpha 2$ helix and V5; mutations at position 350 ($\alpha 2$ helix C-terminal end) and position 460 in V5 correlated with ANAb sensitivity, whereas mutations at position 340 ($\alpha 2$ helix N-terminal end) were correlated with resistance. Additional differences between sensitive and resistant phenotypes not resolved on the crystal structure and, thus, missing from the model include positions in C1 and gp41.

Additionally, there were residues in V1/V2, C2, and gp41 that contained amino acid substitutions and/or PNG locations that correlated with both sensitive and resistant Env variants. Several positions were implicated in affecting the level of NAb sensitivity by multiple mutational mechanisms, sometimes demonstrating conflicting outcomes from the same location.

TABLE 5. Mutations that modulate neutralization phenotype according to point mutations

ANAb phenotype ^a	Amino acid position ^b	Env location ^d	Correlation ^c	P value	Mutation(s)
Sensitive	236	C2	0.460	<0.001	T→S, K
	268	C2	0.472	<0.001	E→G, K
	314	V3	0.460	<0.001	G→R, K
	318	V3	0.460	<0.001	V→T, A
	350	C3	0.473	<0.001	R→Q, S, G, K
	535	gp41 ER	0.473	<0.001	M→V, I, T
Resistant	684	gp41 ER	0.460	<0.001	L→I
	829	gp41 CT	0.469	<0.001	V→I
	49	C1	-0.540	<0.001	T→D, N
	89	C1	-0.460	<0.001	V→L
	142	V1V2	-0.416	<0.001	S→T, N
	143	V1V2	-0.465	<0.001	S→T, D, V
	149	V1V2	-0.531	<0.001	M→I, V
	219	C2	-0.460	<0.001	A→T
	227	C2	-0.460	<0.001	K→R
	234	C2	-0.447	<0.001	N→D, K
	295	C2	-0.460	<0.001	N→E
	297	V3	-0.460	<0.001	T→I
	305	V3	-0.460	<0.001	K→R
	340	C3	-0.453	<0.001	N→E, K, R
	565	gp41 ER	-0.460	<0.001	L→M
	689	gp41 MPER	-0.460	<0.001	V→I

^a The presence of a PNG at these positions is correlated with sensitivity or resistance to ANAb.

^b Numbered with respect to reference strain HXB2.

^c Determined by two-tailed Spearman rank correlation and 95% confidence intervals.

^d gp41 ER, extracellular region of gp41; gp41 MPER, membrane-proximal external region of gp41; gp41 CT, cytoplasmic tail of gp41.

For example, amino acid changes in positions 234 to 236 in C2 correlated with a sensitive phenotype. This sensitivity was associated with the presence of a PNG at 234 in one case and the absence of the same PNG via the presence of T236K in another case. In direct contrast, the removal of the same PNG at 234 by either mutation N234D or N234K correlated with a resistant phenotype. An intact PNG at 295 was found in sensitive Env variants; the removal of the same PNG by mutation N295E or T297I was correlated with resistance to ANAb. Additional positions with multiple mutations that correlated with resistance occurred at position 49, with a T-to-N (PNG addition) or T-to-D mutation, and position 142, with an S-to-N (PNG addition) or S-to-T mutation.

DISCUSSION

Over the last 25 years, our understanding of the nature of NABs in HIV-infected subjects has been informed by accumulating knowledge in several key areas. These include the cloning and characterization of human neutralizing monoclonal antibodies, an understanding of the role of carbohydrate in masking neutralization epitopes, and success in obtaining crystal structures of the primate lentivirus Env protein cores. Much of the early literature focused on the neutralization of laboratory or primary HIV isolates that are unrelated to the subject (5, 6, 29, 32), driven in part by a desire to identify and to characterize those subjects with exceptionally broad HNABs in an effort to clone monoclonal antibodies from these subjects.

FIG. 3. Env residues correlated with susceptibility to ANAb. A structural view of HIV-1 gp120 is shown with the specific position numbers of amino acids and PNG sites correlated with ANAb-resistant Env proteins (left) and ANAb-sensitive Env proteins (right). Residues correlated with resistance are shown in red; residues correlated with sensitivity to ANAb are in green. Amino acid point substitutions are in brighter shades of color, and PNG sites are in darker shades of color. The variable loops are labeled and shown in blue. The core of gp120 is in gray, with the CD4bs in brown. The structure is oriented such that this is the top of gp120, with gp41 (not shown) perpendicular to and intersecting straight into the plane of the image. Additional residues not depicted in this structure but found to be correlated with Env neutralization by ANAb are in C1 and gp41 (Table 4).

Technical challenges in growing primary viruses without laboratory adaptation have limited the study of responses to autologous variants, which was overcome recently by using pseudoviruses made with representative viral envelopes as targets (21). These studies indicate that NAb increase in titer and avidity early in infection and typically show little, if any, neutralization of contemporaneous virus (15, 30). In mother-to-child transmission, both sensitive and resistant variants are present in the quasispecies, and the neutralization-resistant variants are transmitted to the infants (39). The longitudinal analysis of autologous Env variants in chronic infection in individual subjects shows that glycans are added and shifted in a dynamic fashion (38), termed the glycan shield, a finding that extends early (7) and recent (14) work in the simian immunodeficiency virus field and also has been documented in studies of simian-human immunodeficiency virus (1).

Our goal in this study was to gain insight into the ANAb response in HIV controllers with significant HNABs and to examine the changes to the autologous Env proteins. We hoped that this work would augment the earlier studies of the longitudinal development of ANABs and HNABs (10, 22). To our knowledge, the approaches taken in this study have enabled us to examine for the first time the changing character of ANABs against autologous variants in a cohort of long-term, drug-naïve, chronically infected HIV controllers. Similarly to studies of chronically infected mothers (39), both sensitive and resistant Env variants are present in the quasispecies. The results presented in this study show that neutralization is a complex and dynamic process, with varied responses to individual Env proteins.

It is not surprising that the viremic subjects in our study continued to maintain and generate strong ANAb responses

against certain related variants, since all of the subjects are asymptomatic, with preserved CD4⁺ T-cell responses being critical for maintaining the HIV-1-specific B-cell response (24, 27). Published studies of ANABs against the viral quasispecies as a whole early in infection show low levels of contemporaneous neutralization, with increasing titers in later months and years (29). In contrast, we observed that neutralization activity against individual contemporaneous viral variants isolated after many years of infection was present at the matched time point, and these variants frequently were not better neutralized by samples of plasma from later time points. The ANAb response directed to individual variants shows that high levels of antibodies can persist over time against many variants. If maintained long term, Env variant-specific ANABs would require continuous B-cell stimulation by antigen. Cloned variants were unique, and those from different time points clustered independently, suggesting that the sensitive variants are reduced or eliminated from the plasma and that novel changes confer escape upon these Env variants. It is likely that the polyclonal response to these eliminated variants persists due to NABs directed to determinants such as the CD4bs. At the latest time point, some subjects had predominantly escape variants that were resistant to autologous neutralization. In addition, appreciable increases in titer were detected against some variants, supporting the ability of the autologous response to limit certain Env variant populations. Thus, the phylogenetic and ANAb data taken together indicate that viral *env* evolution persists, allowing the escape and development of resistance to ANABs, but other Env sequences continue to remain sensitive to NABs and do not develop resistance in chronically infected viremic and elite controllers.

Controllers in this study, despite having low or undetectable

levels of virus in plasma, have continuous changes in the viral Env sequences over relatively short periods of time. As the result of this dynamic viral evolution, they also have changing ANAb titers. The positive correlation between plasma HIV RNA levels and ANAb titers in controllers suggests an active process that differs in the rate of change per subject. This observation was seen in subjects chronically infected with *nef*-attenuated HIV-1 (36). Increased virus replication may provide the antigenic stimulation necessary to maintain and induce changes in the ANAb response through the maturation of the humoral response due to continuous low-level replication and the resulting heterogeneity of the viral variants (9, 12). The level of broadening in the subjects did not correlate with plasma viremia, percent diversity, or the length of infection (from the time of diagnosis) in our study. This likely is due to the cross-sectional nature of the subject samples analyzed for breadth, making it difficult to determine parameters affecting HNABs, including the time of first detection. Evidence for weak but broad NAb responses in the elite controllers may be indicative of positive ANAb titers at other times in infection that were not analyzed. The greater degree of diversity and breadth in EC1 suggests that the autologous response is even more diverse and vigorous at other time points. Additionally, the earlier priming of B cells and other antibody-secreting cells by the transient low-level viral replication of resistant strains may drive continued antibody responses to later related strains, as documented for other chronic viruses (19, 25). We identified more than 30 unique *env* residues correlated with sensitivity or resistance to ANAb in chronic infection. This was a purely sequence-based analysis of functional Env proteins from five subjects at several time points and required further experimentation to establish the importance of each residue in the context of Env conformation and structure. Nonetheless, the sequence analysis identifies specific amino acids that may be under selective pressure from NABs and points to the regions of Env that impact sensitivity to ANABs, thereby providing the framework for NAb-based vaccine approaches.

Having strong contemporaneous ANAb responses directed against autologous Env variants suggests a role for these responses in contributing to the durable control of HIV replication, as has been suggested by studies of early versus chronic infection (10). Viral evolution and the subsequent production of ANAb clearly continues in long-term chronic infection, consistently with the viral genetic diversity seen in these persons (13), and is an indication that NABs are partially responsible for driving this variation. The presence of a dynamic autologous response, indicated by alterations in neutralization of a variant over time, was accompanied by changes in the heterologous response to particular virus isolates over time. Although the overall level of the neutralization of the number of heterologous viruses neutralized did not change appreciably, the HNAB titers changed as the ANAb response evolved in response to altered autologous Env variants presented to the humoral system. The broadening of the response therefore may be viewed as a by-product of the individual's own autologous response generated against the milieu of autologous Env conformations, which in turn is a dynamic response, not simply an additive effect. However, the development of HNABs, while commonly found in viremic subjects, is neither inevitable nor

predictable. Understanding the mechanism of HNAB development remains a key objective for vaccine design.

ACKNOWLEDGMENTS

We gratefully acknowledge the critical review and helpful comments of J. Overbaugh, M. Martin, W. Blay Puryear, and L. Stamatatos during the preparation of the manuscript.

The work was supported by PHS grant NIH-P01-AI054564, the Immunology Core of the Center for AIDS and STD at the University of Washington (P30 AI27757), and generous gifts from the James B. Pendleton Charitable Trust and the Murdock Charitable Trust.

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