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Neutralizing Antibodies Do Not Mediate Suppression of Human Immunodeficiency Virus Type 1 in Elite Suppressors or Selection of Plasma Virus Variants in Patients on Highly Active Antiretroviral Therapy

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Neutralizing antibodies (NAb) against autologous virus can reach high titers in human immunodeficiency virus type 1 (HIV-1)-infected patients with progressive disease. Less is known about the role of NAb in HIV-1-infected patients with viral loads of <50 copies/ml of plasma, including patients on effective highly active antiretroviral therapy (HAART) and elite suppressors, who control HIV-1 replication without antiretroviral therapy. In this study, we analyzed full-length *env* sequences from plasma viruses and proviruses in resting CD4$^+$ T cells of HAART-treated patients, elite suppressors, and untreated HIV-1-infected patients with progressive disease. For each patient group, we assessed plasma virus neutralization by autologous, contemporaneous plasma. The degree of *env* diversity, the number of N-linked glycosylation sites, and the lengths of variable loops were all lower in elite suppressors than in HAART-treated and untreated viremic patients. Both elite suppressors and HAART-treated patients had lower titers of NAb against HIV-1 lab strains than those of untreated viremic patients. Surprisingly, titers of NAb against autologous, contemporaneous plasma viruses were similarly low in chronic progressors, elite suppressors, and HAART-treated patients. In elite suppressors and HAART-treated patients, titers of NAb against autologous plasma viruses also did not differ significantly from titers against autologous proviruses from resting CD4$^+$ T cells. These results suggest that high-titer NAb are not required for maintenance of viral suppression in elite suppressors and that NAb do not select plasma virus variants in most HAART-treated patients. Both drug-mediated and natural suppression of HIV-1 replication to levels below 50 copies/ml may limit the stimulation and maintenance of effective NAb responses.

Although antibodies control many viral infections, human immunodeficiency virus type 1 (HIV-1) replicates continuously in the face of a strong antibody response (6). Envelope (Env) spikes on the surfaces of HIV-1 virions resist antibody binding through occlusion of epitopes within the trimeric structure (33, 61), extension of variable loops from the surface of the protein (23, 24), steric and conformational blocking of receptor binding sites (8, 29, 30), and extensive glycosylation (48, 59, 61). Structural features of HIV-1 gp120, particularly its variable loops, allow it to tolerate a vast array of mutations without a loss of function (24). This allows repeated escape from neutralizing antibody (NAb) responses that do develop. NAb escape has been demonstrated in culture assays (36, 49, 62) and animal models (5). One notable study showed that cocktails of NAb against conserved Env epitopes exert little control on established HIV-1 infection in the SCID-Hu mouse model of HIV infection (47).

Several groups have shown that NAb against autologous virus develop within months of seroconversion in acutely infected patients (1, 19, 50, 59). Although these NAb ultimately reach fairly high titers, escape mutants are selected rapidly due to their high levels of ongoing viral replication (50, 59). Other studies have shown that chronically HIV-1-infected patients also develop NAb against earlier viral isolates but show little neutralization of contemporaneous virus (53, 55).

Less is known about the role of NAb in HIV-1-infected patients with viral loads of <50 copies/ml of plasma, including elite suppressors (ES) and patients on effective highly active antiretroviral therapy (HAART). ES are a distinct subset of long-term nonprogressors (LTNPs) who maintain stable CD4$^+$ T-cell counts and viral loads of <50 copies/ml without antiviral therapy. Cytotoxic T-lymphocyte (CTL) responses appear to play a significant role in viral suppression in these individuals, as the major histocompatibility complex class I allele group HLA-B*57 is overrepresented in this group (2, 37). It is not known whether NAb also play a significant role in viral suppression in this population. Most previous studies of NAb in LTNPs have concluded that LTNPs generally have higher-titer NAb responses against lab strains and heterologous primary isolates of HIV-1 than chronically infected individuals with progressive disease (7, 39, 42, 45). However, one study found that LTNPs with relatively low viral loads had weak NAb titers against primary isolates (21), and a study of autologous neutralization in three LTNPs showed little initial neutralization of autologous virus, with somewhat higher NAb titers developing over time (4). These studies were done in individuals with a wide range of viral loads, and none looked specifically at

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the ES subset of LTNPs, who maintain viral loads of <50 copies/ml of plasma without therapy.

The second group of individuals who maintain viral loads of <50 copies/ml are patients on suppressive HAART. Although the viral load may be below the limit of detection of ultrasensitive clinical assays in these patients, free virus is consistently detectable in the plasma by reverse transcription-PCR (RT-PCR) assays sensitive to <50 copies of viral RNA/ml (15, 22, 41). It is currently unclear how suppression affects the NAb response against autologous virus in these individuals. Previous studies have examined neutralizing antibody responses in HAART-treated patients against earlier autologous viral isolates (3, 28), but never against a range of viral isolates from contemporaneous plasma. It is thus possible that NAb play a role in selecting the plasma virus variants detectable in patients on HAART.

In order to study the role of NAb in ES and HAART-treated patients, we characterized the *env* genes of the plasma virus quasispecies and measured NAb responses in nine ES, nine HAART-treated patients, and seven untreated, chronically HIV-1-infected patients with progressive disease (chronic progressors). The diversity and characteristics of the *env* sequences in the residual plasma virus quasispecies have not previously been investigated with either ES or patients on HAART with viral loads of <50 copies/ml. We measured HIV-1 binding antibody titers as well as titers of NAb against the neutralization-sensitive lab strains SF162 and NL4-3 in all study groups. Using pseudoviruses produced from cloned patient *env* genes, we measured titers of NAb in each patient group against autologous, contemporaneous plasma virus. Finally, in ES and HAART-treated patients, we measured titers of NAb against latent proviruses archived in resting $CD4^+$ T cells. The results of this study provide insight into the role of antibody responses in patients with suppression of viremia to <50 copies/ml of plasma.

MATERIALS AND METHODS

Patient selection. Chronic progressors were patients who were ART naïve and had viral loads above 10,000 copies/ml of plasma. Six of seven chronic progressors had $CD4^+$ T-cell counts between 200 and 500 cells/μl. HAART-treated patients were HIV-1-infected adults who had evidence of disease progression prior to treatment with antiretroviral drugs and who had maintained viral loads of <50 copies/ml of plasma for at least 6 months on HAART. The criteria for ES were as follows: subjects had positive HIV-1 Western blots but maintained suppression of viremia to <50 copies/ml without antiretroviral therapy. The salient clinical features of the patients are shown in Table 1. Informed consent was obtained prior to phlebotomy. The protocol was approved by an institutional review board of the Johns Hopkins University School of Medicine. Blood was anticoagulated with citrated dextrose and centrifuged on Ficoll-Hypaque gradients to separate plasma and peripheral blood mononuclear cells.

Plasma HIV-1 RNA quantification. Viral RNA was quantified using the ultrasensitive Roche Amplicor Monitor system, version 1.5 (Roche Molecular Systems, Inc., Blanchburg, New Jersey), which has a lower limit of quantification of 50 copies/ml.

Detection of binding antibody. Binding antibody was measured using the Vironostika HIV-1 Microelisa system (Biomerieux), with an initial plasma dilution of 1:75 followed by seven fivefold serial dilutions for chronic progressors and five fivefold serial dilutions for ES and HAART-treated patients. Half-maximal binding was calculated for each plasma sample, based on a growth curve fit to each data set in Microsoft Excel.

Genomic DNA and viral RNA isolation. Magnetic bead depletion was performed on peripheral blood mononuclear cells to enrich for resting $CD4^+$ T cells as described previously (26). Genomic DNAs were purified from resting $CD4^+$ T cells by use of a Puregene kit (Gentra). Ten to 20 ml of plasma from each patient was ultracentrifuged at 25,200 × *g* for 2 h at 4°C to concentrate plasma virions. Viral RNAs were isolated from pelleted virions using a QIAGEN viral RNA isolation kit.

***env* amplification.** *env* was amplified from provirus genomic DNA by limiting-dilution "digital" nested PCR. An outer reaction was performed with the primers 5′ *env* out (ATG GCA GGA AGA AGC GGA GAC AG) and RT4.2 (GCT CAA CTG GTA CTA GCT TGA AGC ACC). A nested reaction was then performed with the primers 5′ *env* inner (GAT AGA CGC GTA GAA AGA GCA GAA GAC AGT GGC AAT G) and 3′ *env* inner (CCT TGT GCG GCC GCC TTA AAG GTA CCT GAG GTC TGA CTG G). These primers, except RT4.2, were previously described (43). All PCRs were performed with Accuprime *Pfx* polymerase (Invitrogen) in order to maximize fidelity. PCRs were performed at a concentration of DNA that would produce <1/3 positive PCRs. PCR products were gel purified using a QIAquick gel extraction kit (QIAGEN, Valencia, CA) and directly sequenced using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Chromatograms were examined manually for the presence of double peaks indicative of two templates per sequencing reaction. Such sequences were discarded.

env was amplified from viral RNA by a two-step RT-PCR protocol. Control reactions run without reverse transcriptase were invariably negative. cDNA was produced using the RT4.2 primer and SuperScript II polymerase (Invitrogen). Nested PCR was then performed using the same primers used for genomic DNA. For chronic progressors, PCRs were performed with cDNAs at a limiting dilution, and the products were directly sequenced. For ES and HAART-treated patients, PCRs were performed with cDNAs near the limiting dilution, and products were cloned as described below prior to being sequenced. To avoid template resampling, only products of independent PCRs were considered independent viral clones (32). Sequences were assembled using CodonCode Aligner, version 1.3.1, and aligned using ClustalX. Alignments were manually adjusted in Bioedit. Gaps were stripped from the alignments prior to calculations of diversity. Plasma virus *env* gene diversity was calculated only for individuals from whom at least three independent *env* genes were amplified. Proviral clones with G-to-A hypermutation were not included in diversity analysis or analysis of N-linked glycosylation. Diversity was calculated using the Kimura two-parameter model (27) in Mega, version 3.1. The number of N-linked glycans was predicted using N-glycosite (Los Alamos National Laboratory).

Cloning and pseudovirus production. *env* genes were cloned into the PCI-Pre vector as described previously (43). The resulting vectors were cotransfected, along with a previously described *env*-deleted NL4-3 green fluorescent protein reporter virus (44), into 293T cells. *env* genes from patients on HAART were cotransfected with one of two reporter viruses bearing multiple antiretroviral drug resistance mutations to limit the effects of residual antiretroviral drugs in the test plasma. 293T cell supernatants were harvested after 48 h, centrifuged to remove debris, and snap frozen.

Immunoglobulin depletion with protein A. Immunoglobulins were depleted from 200-μl aliquots of heat-inactivated plasma using a NAb-protein A spin purification kit (Pierce). Immunoglobulins were eluted from protein A using the supplied elution buffer, and the pH was neutralized with neutralization buffer. Immunoglobulin-depleted plasmas and eluted immunoglobulins were used in neutralization experiments for two patients from each study group. Immunoglobulins depleted from normal human plasma were used as a control.

Neutralization assay. Neutralization assays were performed essentially as described previously (59). Briefly, pseudoviruses were titrated on TZM b1 cells (George Shaw, NIH AIDS Research and Reference Reagent Program) to determine a linear range of infection for each pseudovirus. Infections were then performed in duplicate with a concentration of virus within this linear range, along with serial dilutions of patient plasma that had been heat inactivated at 56°C for 30 min. All assays were performed in the presence of 10% total human plasma. Each virus was preincubated with 5% test plasma and with four 2.5-fold serial dilutions of test plasma in normal human plasma. To determine neutralization, each test plasma well was compared to wells containing an equal concentration of normal human plasma. Each patient plasma was also tested for neutralization of HIV-1 pseudotyped with the vesicular stomatitis virus glycoprotein (VSV G) envelope to rule out nonspecific neutralization, particularly by residual drugs in the plasmas of patients on HAART. The effect of test plasma on infection by VSV G-pseudotyped virus was a <20% enhancement or inhibition of infection at the highest concentration of test plasma. Neutralization or enhancement of infection by VSV G-pseudotyped virus was used to correct the neutralization values obtained for HIV-1 *env*-pseudotyped viruses with those obtained for plasmas. Virus-antibody mixtures were incubated for 1.5 h at 37°C and then added to TZM b1 cells which had been seeded into 96-well plates 12 h previously. Infections were performed in the presence of 40 μg/ml DEAE-dextran. Infection was measured after 48 h by determining luciferase production.

Statistics. Fifty percent inhibitory concentrations (IC_{50}s) were calculated based on a growth curve fit to each neutralization assay in Microsoft Excel. The significance of all comparisons was calculated using Student's two-tailed *t* test. Viruses with IC_{50}s of >0.05 were assigned a value of 0.05 for statistical analysis.

TABLE 1. Patient characteristics

Patient category and no.	Date (mo/yr or yr only) of diagnosis	Date (mo/yr) of sampling		Plasma HIV RNA (copies/ml) at latest plasma sampling date	CD4 count (cells/μl) at latest plasma sampling date	CD4 nadir[a] (cells/μl)	Date (mo/yr) of initiation of suppressive HAART[b]	Gender[c]
		Plasma	$CD4^+$ T cells[d]					
Chronic progressors								
C61	9/99	9/04		19,100	1,261			F
C62	1998	9/04		33,300	481			M
C93	2/01	3/05		4,7270	402			M
C94	12/99	2/05		22,898	351			M
C96	8/01	3/05		12,500	400			M
C98	9/04	3/05		17,838	426			F
C109	2002	5/05		61,000	222			F
HAART-treated patients								
H9	1984	12/03	8/04	<50	800	132	10/02	M
H22	1987	4/04	10/04	<50	1,174	222	10/01	F
H23	11/00	4/04	10/04	<50	571	361	3/01	M
H25	5/01	4/04	4/04 5/05	<50	558	95	6/01	M
H26	7/99	4/04	4/05	<50	624	200	5/00	M
H28	1989	5/03	5/05	<50	543	211	10/98	M
H135	1990	6/04	12/03	<50	384	65	8/98	F
H148	1987	6/04	4/04	<50	638	33	6/98	F
H154	6/86	7/04	7/04	<50	223	18	4/02	M
Elite suppressors								
ES2	1986	5/04	9/04 5/05	<50	383			F
ES3	1991	3/04	7/04	<50	677			F
ES4	1996	8/04	8/04	<50	837			F
ES5	1990	8/04	8/04	<50	704			F
ES6	1992	7/04	7/04	<50	773			F
ES7	1994	1/05	1/05 5/05	<50	1,125			M
ES8	2003	6/04 9/04	4/05	<50	458			M
ES9	1999	3/04 8/04	2/05	<50	800			F
ES10	2002	3/04		<50	900			F

[a] Lowest CD4 count before initiation of effective therapy.
[b] Date the patient began an effective HAART regimen that led to suppression of viremia to <50 copies/ml.
[c] F, female; M, male.
[d] Resting $CD4^+$ T-cell sequences were not analyzed in chronic progressors because in viremic patients, most of the viral genomes in resting $CD4^+$ T cells are labile unintegrated forms that resemble plasma virus.

Nucleotide sequence accession numbers. The sequences determined for this study have been submitted to GenBank under accession numbers DQ410040 to DQ410649.

RESULTS

Full-length *env* sequences can be amplified from plasma viruses of ES and patients with suppression of viremia by HAART. Little is known about the role of NAb in ES and patients with suppression of viremia by HAART because it is difficult to isolate plasma virus when the viral load is <50 copies/ml. Since NAb against autologous virus can exert strong selective pressure in viremic patients (50, 59), it is possible that NAb also play a role in the suppression of viral replication in ES and in the selection of plasma variants in patients on HAART. To address these issues, we first amplified multiple full-length *env* genes from plasma viruses of seven chronic progressors. Full-length *env* genes from patients on HAART and ES proved extremely difficult to amplify from plasma, requiring the development of an ultrasensitive RT-PCR method. With this method, we amplified *env* genes from free viruses in the plasmas of patients on HAART, confirming previous studies detecting low-level plasma viremia in these individuals (17, 22, 51). In addition, we were able to amplify *env* genes from plasma viruses in eight of nine ES. For ES8 and ES9, plasma virus *env* was amplified from plasma at two time points. Phylogenetic analysis demonstrated that all *env* sequences were patient specific. These results demonstrate that, like patients on HAART, most ES have continuous, extremely low-level viremia. *env* genes were also amplified from proviruses in resting $CD4^+$ T cells from all HAART-treated patients and the eight ES for whom cells were available. Proviruses archived in resting $CD4^+$ T cells constitute a stable reservoir of virus which can persist for years, despite antiretroviral therapy or immune responses (10–12, 16, 60). This latent reservoir may therefore include viral variants that were archived at earlier stages of infection (22). *env* genes were not amplified from proviruses in resting $CD4^+$ T cells of chronic progressors, since most cell-associated virus in viremic patients is the product of recent infection and does not differ significantly from plasma virus (38).

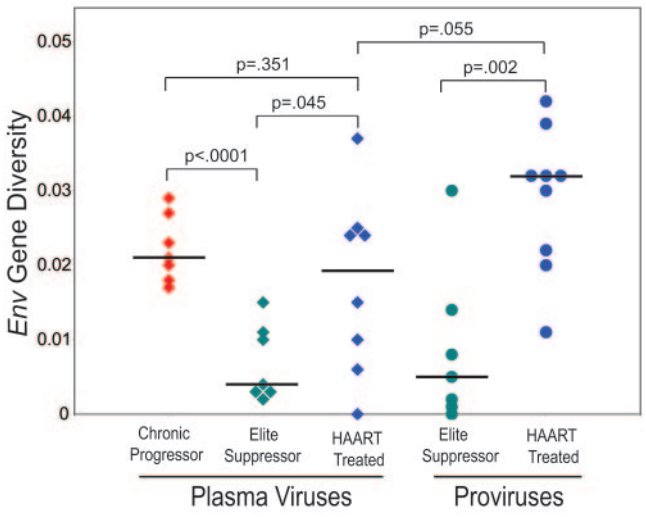

FIG. 1. Intrapatient *env* gene diversity. Diversity was calculated for each patient's viral quasispecies as the average genetic distance between full-length *env* clones by the two-parameter (Kimura) method. Analysis was performed on plasma virus *env* genes from chronic progressors. Plasma virus *env* sequences were distinct from proviral sequences in both ES and HAART-treated individuals, so plasma virus *env* and proviral *env* were analyzed separately for these study groups. Diversity was calculated only for individuals from whom at least three independent *env* genes were amplified from plasma viruses or proviruses. Each diamond represents the diversity of *env* genes amplified from plasma viruses of one individual. Each circle represents the diversity of *env* genes amplified from proviruses in resting $CD4^+$ T cells of one individual. Horizontal hash marks indicate the median diversity for each study group.

We amplified and sequenced a median of 15 independent full-length *env* clones from the plasma of each chronic progressor, 9 from the plasma and 9 from the resting $CD4^+$ T cells of each ES, and 6 from the plasma and 30 from the resting $CD4^+$ T cells of each HAART-treated patient. Sequences from two ES8 and two ES9 plasma time points were included in all *env* analyses. In total, 611 completely independent full-length *env* clones from 25 study subjects were sequenced.

Diversity of plasma virus and proviral *env* sequences is lower in ES than in chronic progressors and patients with suppression of viremia by HAART. The diversity of the viral quasispecies may be a key determinant of the development and maintenance of NAb against autologous virus (47). In a study of patients undergoing structured treatment interruption, lower diversity prior to treatment correlated with better autologous virus neutralization and better viral suppression after HAART interruption (25). The diversity of *env* sequences of plasma viruses from ES has not been previously characterized. To quantitate intrapatient diversity, we calculated the average genetic distance by the Kimura method (27) between all *env* genes amplified from each individual. *env* sequences amplified from plasma viruses and from resting $CD4^+$ T-cell proviruses were analyzed separately. We found that the *env* diversity of plasma viruses from ES was significantly lower than the diversity of plasma viruses from chronic progressors, even when two different time points for ES8 and ES9 were included in the analysis (median, 0.4% versus 2.1%; $P < 0.0001$) (Fig. 1). The diversity of ES plasma viruses was also significantly lower than that of plasma viruses from HAART-treated patients (median, 0.4% versus 1.95%; $P = 0.045$). As with the plasma virus *env* sequences, archived proviral *env* sequences in resting $CD4^+$ T cells of ES showed significantly less diversity than proviral *env* sequences from HAART-treated patients (median, 0.5% versus 3.2%; $P = 0.002$). Taken together, these results demonstrate that ES viruses have relatively low overall *env* diversity in both the plasma and the resting $CD4^+$ T-cell provirus pools.

In contrast to that of the plasma virus *env* genes from ES and chronic progressors, the diversity of plasma virus *env* sequences from patients on HAART varied greatly between patients, ranging from 0% to 3.7% (Fig. 1). This may be explained by a recent finding that the majority of plasma virus sequences in some patients with suppression of viremia by HAART fall into a single dominant phylogenetic taxon (J. R. Bailey et al., submitted for publication). Overall, however, the *env* diversity of plasma viruses from HAART-treated patients was not significantly lower than the diversity of proviruses from the same patients. The *env* diversity of plasma viruses and proviruses in HAART-treated patients was also not significantly different from the diversity of *env* sequences in chronic progressor plasma viruses. Taken together, these results suggest that high levels of viral diversity are maintained in the latent reservoir in patients on HAART and that diverse viruses are also present in the plasmas of most individuals.

Predicted N-linked glycan number and V1-V5 length are lower for ES than for chronic progressors or patients with suppression of viremia by HAART. The number of N-linked glycans in the V1-V5 region of *env* and the length of the V1-V5 region are thought to be major determinants of sensitivity to neutralization by both autologous and heterologous antibodies (9, 13, 14, 18, 46). The number of predicted N-linked glycosylation sites in V1-V5 of *env* and the V1-V5 length were not significantly different between patients on HAART and chronic progressors (Fig. 2A and B). Viruses in patients on HAART likely experienced significant antibody pressure targeting the *env* gene prior to viral suppression by HAART. Variable loop lengths and glycosylation levels are apparently maintained despite viral suppression. This could be the result of viral archiving in the latent reservoir or of continued antibody pressure on *env* during HAART. As shown in Fig. 2A and B, levels of N-linked glycosylation and V1-V5 lengths of both plasma virus *env* and proviral *env* were significantly lower and shorter, respectively, for ES than for either chronic progressors or patients on HAART. Deletions and low glycan densities were not focused at any particular variable region. Generally, shorter variable loops and low levels of glycosylation suggest that the virus in these individuals may have experienced relatively little antibody pressure targeting *env* throughout the course of infection.

HIV-1 binding antibody titers are lower in ES and HAART-treated patients than in chronic progressors. To assess the relative antibody levels in plasma from the three patient populations, we first measured anti-HIV-1 binding antibody titers using an enzyme-linked immunosorbent assay that detects binding antibodies against proteins from lysed HIV-1 virions. ES had significantly lower titers of anti-HIV-1 binding antibodies than did chronic progressors (median, 1:8,280 versus 1:71,966; $P = 0.007$) (Fig. 3). This was also true of the HAART-

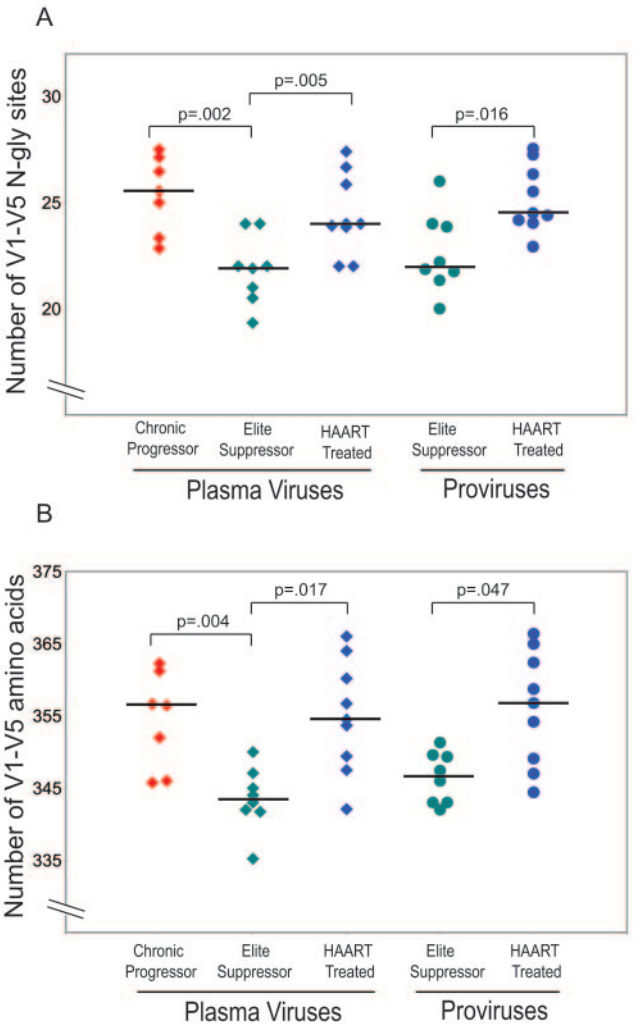

FIG. 2. Predicted numbers of N-linked glycans and numbers of amino acids in the V1-V5 region of *env* from chronic progressors, ES, and HAART-treated patients. (A) Numbers of predicted N-linked glycans in V1-V5. Each diamond represents the mean number of predicted V1-V5 N-linked glycans for all *env* clones amplified from the plasma of one individual. Each circle represents the mean number of predicted V1-V5 N-linked glycans for all proviral *env* clones amplified from resting $CD4^+$ T cells from one individual. Horizontal hash marks indicate the median N-linked glycan number for each study group. (B) Numbers of amino acids in the V1-V5 region of *env*. Each diamond represents the mean number of V1-V5 amino acids for all *env* clones amplified from the plasma of one individual. Each circle represents the mean number of V1-V5 amino acids for all proviral *env* clones amplified from resting $CD4^+$ T cells from one individual. Horizontal hash marks indicate the median V1-V5 length for each study group.

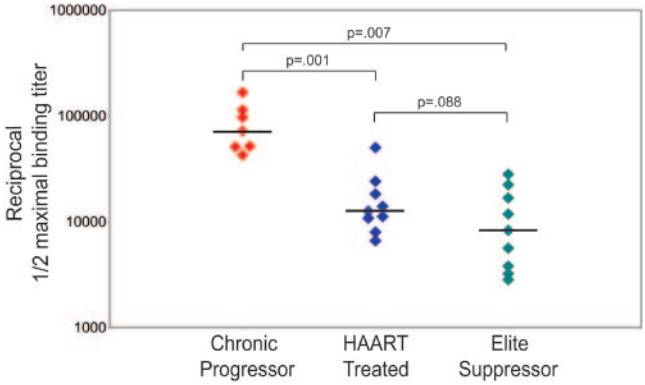

FIG. 3. Binding antibody titers against HIV-1. Each data point indicates the reciprocal of the plasma dilution at which ELISA binding of antibody to lysed HIV-1 virion proteins was one-half the maximum. Horizontal hash marks indicate the median titer for each patient group.

treated patients (median titer, 1:12,605 for HAART-treated patients versus 1:71,966 for chronic progressors; $P = 0.001$). The relatively low titers of HIV-1 binding antibody in ES and HAART-treated patients likely reflect the low levels of antigenic stimulation due to the suppression of viral replication in both groups.

Titers of Nab against neutralization-sensitive lab strains are lower in ES and HAART-treated patients than in chronic progressors. To measure titers of NAb capable of inhibiting HIV-1 infection of target cells in vitro, we used a pseudovirus system similar to those that have been used recently to evaluate NAb responses in acute seroconvertors (50, 59). *env* clones were cotransfected with an *env*-deleted NL4-3 reporter virus clone to generate pseudoviruses, which could be tested for neutralization by antibody in single-round infectivity assays (63). We first measured titers of NAb against HIV-1 subtype B lab strains SF162 and NL4-3. Since SF162 and NL4-3 are relatively neutralization sensitive, they serve as an initial screen for heterologous or broadly neutralizing antibodies (31). As shown in Fig. 4A, high-titer NAb against SF162 were detected in chronic progressors, confirming that NAb, if present, are readily detectable in this system. ES had significantly lower titers of NAb against SF162 than those of chronic progressors (median IC_{50}, 1:173 for ES and 1:5,446 for chronic progressors; $P = 0.012$). HAART-treated patients also had significantly lower titers of NAb against SF162 than those of chronic progressors (median IC_{50}, 1:345 for HAART-treated patients and 1:5,446 for chronic progressors; $P = 0.046$). As shown in Fig. 4B, no ES or HAART-treated patients showed any detectable NAb activity against NL4-3, but four of seven chronic progressors showed NAb, with IC_{50} titers ranging from 1:100 to 1:270. Since titers of NAb against SF162 and NL4-3 were even lower in ES than in chronic progressors, it is unlikely that broadly neutralizing antibodies are a key to suppression in ES. The lower titers of NAb in HAART-treated patients than in chronic progressors also suggest that high levels of antigenic stimulation may be required to maintain the NAb against lab strains commonly detected in viremic patients with progressive disease (35, 40, 46).

Very low titers of NAb against autologous, contemporaneous plasma virus were detectable in all chronic progressors. To measure titers of NAb against autologous, contemporaneous plasma virus, we selected multiple *env* clones from each subject for phenotypic study. Given the high viral diversity detected in chronic progressors and HAART-treated patients, it was not possible to study each *env* variant that had been amplified and sequenced. We attempted to obtain a representative sample, however, by choosing clones that differed in their genetic distance from the subtype B consensus and in their variable loop length. These clones were cotransfected with an *env*-deleted NL4-3 reporter virus clone to generate pseudoviruses. The

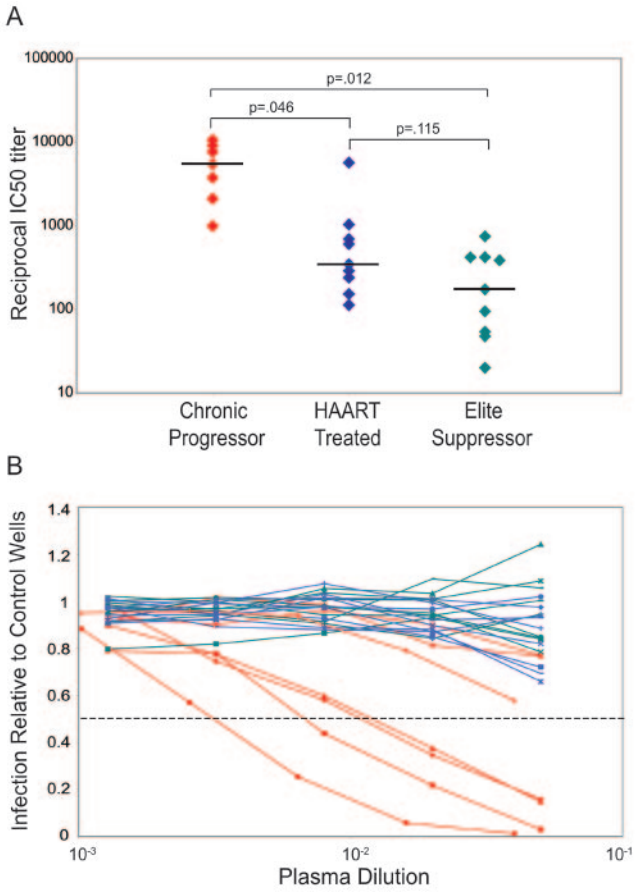

FIG. 4. Neutralizing antibody titers against neutralization-sensitive HIV-1 subtype B lab strains. (A) Neutralizing antibody titers against pseudovirus with strain SF162 Env. The titer is the reciprocal of the plasma dilution at which infection was 50% of the level measured in wells with control plasma (IC_{50}). Each data point indicates the reciprocal IC_{50} of an individual patient plasma against SF162 pseudovirus. Horizontal hash marks indicate the median reciprocal IC_{50} for each study group. (B) Infectivities relative to control of pseudovirus with NL4-3 Env in the presence of serial dilutions of plasma from each study subject. The dashed line indicates a level of infection that is 50% of that seen in control wells with uninfected human plasma. Red lines indicate neutralization by plasmas from chronic progressors. Green lines indicate neutralization by plasmas from ES. Blue lines indicate neutralization by plasmas from HAART-treated patients.

majority of pseudoviruses produced from all three patient groups were functional for single-round infections of target cells, providing evidence that, excluding some proviruses with G-to-A hypermutation, the *env* gene is not defective in most viruses from ES and HAART-treated patients.

Pseudoviruses with Envs from the patients on HAART were produced by using an *env*-deleted NL4-3 reporter virus with multiple antiretroviral drug resistance mutations. This was done to limit the effect of residual drug in test plasmas from these patients. In addition, all plasma samples were tested for neutralization of HIV-1 pseudotyped with the VSV G envelope in order to rule out nonspecific or drug-mediated neutralization of HIV-1. Finally, protein A treatment of plasmas from two subjects in each study group reduced or eliminated neutralizing activity, and immunoglobulin subsequently eluted from protein A neutralized autologous virus (not shown). Thus, the neutralization observed in these experiments was likely to result from the specific binding of antibodies to the HIV-1 Env protein.

We measured titers of NAb in plasmas from chronic progressors, ES, and HAART-treated patients against pseudoviruses produced with autologous plasma virus *env* that was contemporaneous with the plasmas being tested. The measurement of titers of NAb against contemporaneous plasma virus is necessary to determine whether NAb are directly responsible for viral suppression. Low titers of NAb against contemporaneous plasma virus may be due to either generally poor NAb responses or the selection of NAb escape variants by strong NAb responses. In viremic patients, low titers of NAb against autologous, contemporaneous plasma virus are generally due to viral escape (50, 59). To distinguish between these possibilities for ES and HAART-treated patients, we measured titers of NAb against archived proviruses as well as circulating plasma viruses. Proviruses can remain latent in resting $CD4^+$ T cells for a long time, evading immune responses and antiviral therapy (10–12, 16, 60). For this reason, proviral variants, unlike plasma virus variants, may persist indefinitely, despite a strong strain-specific NAb response. Thus, high-titer NAb responses against archived proviral variants would provide evidence of antibody responses against earlier viral variants.

Most plasma-derived *env* sequences were tested for neutralization by autologous, contemporaneous plasma. Since *env* was amplified at two time points for patients ES8 and ES9, some pseudoviruses from these individuals that were not contemporaneous with the test plasmas were also examined. Since proviruses archived in resting $CD4^+$ T cells probably experience very little evolution over short periods of time (17, 51), pseudoviruses produced with proviral *env* sequences were tested with the same autologous plasmas used to test plasma-derived *env* sequences. Using this pseudovirus system, a median of five plasma-derived *env* sequences from each chronic progressor, three from each ES, and two from each HAART-treated patient were tested. A median of one provirus-derived *env* sequence from each ES and four sequences from each HAART-treated patient were tested. Relatively fewer clones were tested from ES because the viral diversity was very low in these individuals. In total, 130 clonal pseudoviruses from 25 different patients were tested for autologous neutralization.

Neutralization of plasma viruses from chronic progressors by autologous, contemporaneous plasma was measured to establish typical NAb titers in individuals with relatively ineffective immune responses to HIV-1. Neutralization of plasma virus was detectable in all of the chronic progressors studied, although median IC_{50} titers were much lower than those observed for neutralization of the sensitive lab strain (SF162) (Fig. 4A). Autologous neutralization titers varied between patients, ranging from >1:20 to 1:189 (Fig. 5A and Table 2). Five of seven chronic progressors showed nearly equivalent neutralization of all *env* variants amplified from the same plasma, even though the clones had different variable loop lengths and many amino acid differences. These results suggest that NAb may exert some selective pressure on HIV-1 *env* in these individuals, although this pressure is not sufficient for effective control of viral replication.

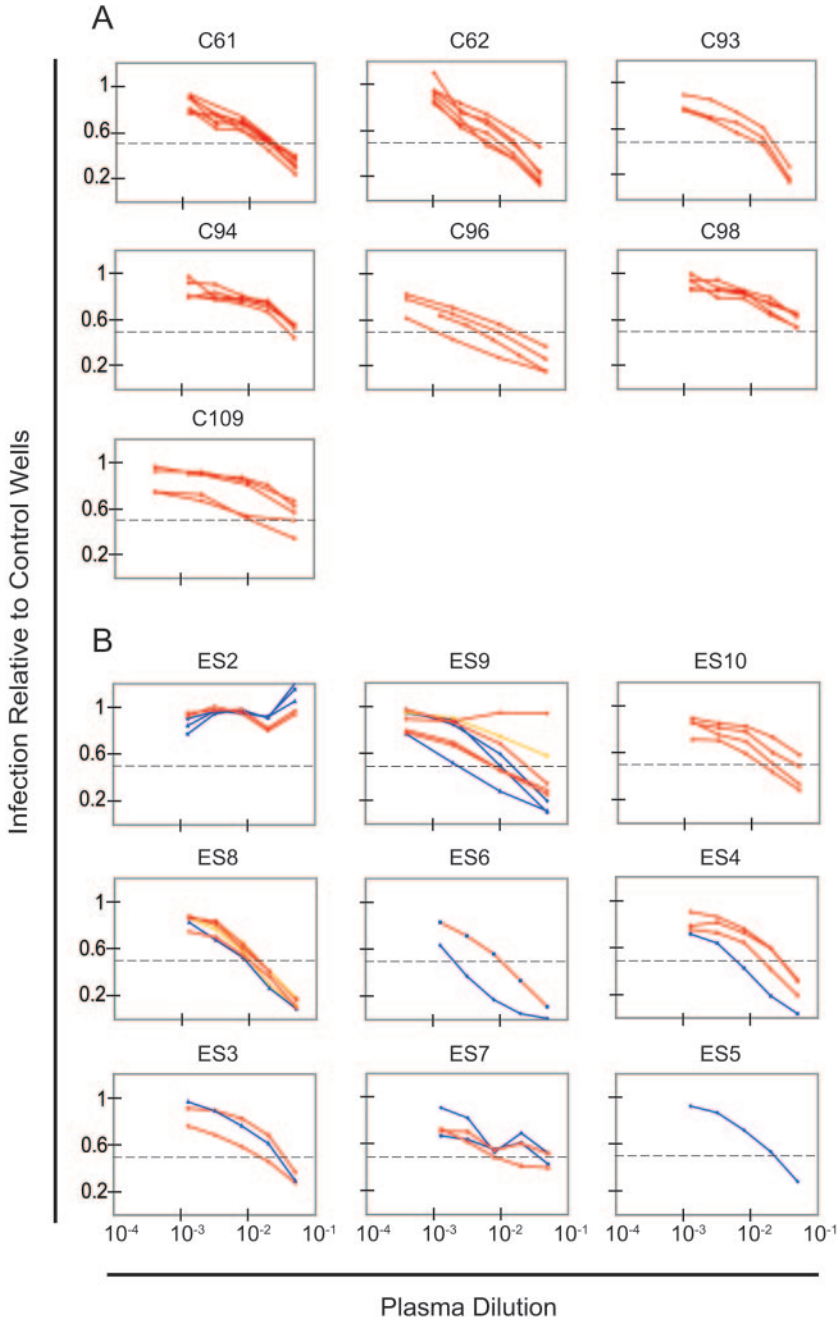

FIG. 5. Autologous neutralization of pseudoviruses with *env* genes from plasma virus or provirus by serial dilutions of chronic progressor, elite suppressor, and HAART-treated patient plasmas. Dashed lines indicate a level of infection that is 50% of that seen in control wells with uninfected human plasma. (A) Infectivities relative to control of clonal pseudoviruses with plasma virus *env* from chronic progressors in the presence of serial dilutions of autologous, contemporaneous plasma. (B) Infectivities relative to control of pseudoviruses with *env* from ES in the presence of serial dilutions of autologous plasma. Pseudoviruses with plasma virus *env* were tested for neutralization by autologous, contemporaneous plasma (red lines) or autologous, noncontemporaneous plasma (orange lines). Proviral *env* pseudoviruses (blue lines) were tested for neutralization by autologous plasma. One *env* variant tested from ES6 (red line with blue points) was amplified independently from both plasma virus and provirus. (C) Infectivities relative to control of pseudoviruses with *env* from HAART-treated patients in the presence of serial dilutions of autologous plasma. Pseudoviruses with plasma virus *env* (red lines) were tested for neutralization by autologous, contemporaneous plasma. Proviral *env* pseudoviruses (blue lines) were tested for neutralization by autologous plasma.

Neutralization of autologous, contemporaneous plasma virus was weak in all ES and undetectable in some individuals. We next examined the neutralization of autologous, contemporaneous plasma viruses in ES. Given the low viral diversity in ES, the pseudoviruses tested for neutralization in this group provide a fairly comprehensive picture of autologous neutralization in these subjects. The results are shown in Fig. 5B and Table 2. ES2 showed no detectable neutralization of contem-

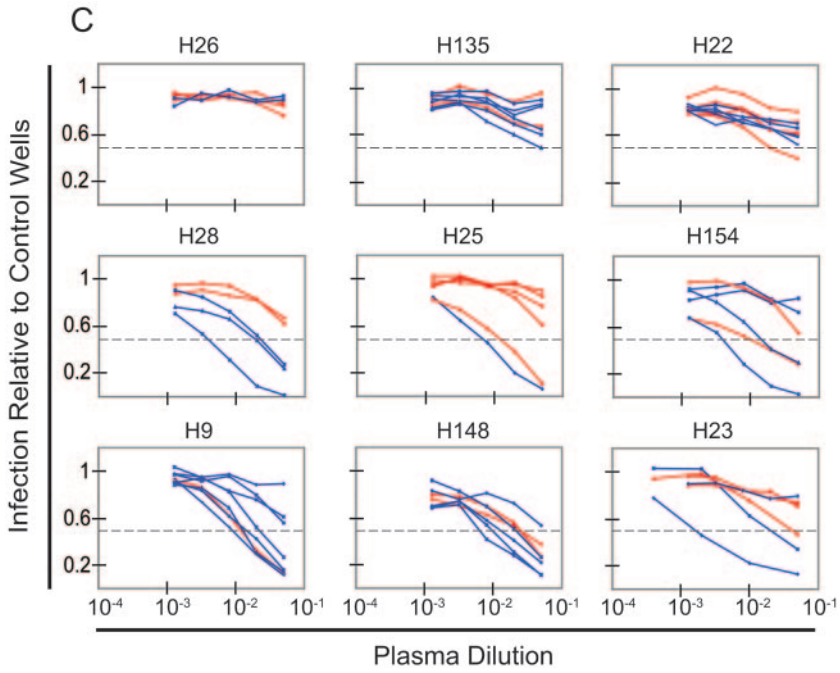

FIG. 5—*Continued.*

poraneous plasma viruses or proviral variants, even at a 1:20 plasma dilution. ES9 showed no detectable neutralization of one plasma virus variant, while several other plasma virus variants amplified from the same plasma sample were neutralized, with IC_{50} titers ranging from 1:44 to 1:172. This individual showed relatively better neutralization of proviral variants, with titers ranging from 1:108 to 1:455. ES10 also showed a range of weak responses to four contemporaneous plasma virus isolates, with one isolate neutralized <50% at a 1:20 dilution of plasma. As shown in Fig. 5B, three subjects, ES8, ES6, and ES4, showed mean IC_{50}s against plasma viruses of 1:107, 1:119, and 1:58, respectively. Two of these subjects, ES6 and ES4, showed higher titers of NAb against proviral variants than against plasma virus variants (provirus IC_{50}s, 1:500 and 1:204), suggesting that titers in these subjects may have reached sufficient levels to drive the selection of NAb escape mutants. Even in these subjects, however, autologous, contemporaneous titers of NAb against plasma viruses were not higher than the highest titers seen in chronic progressors. Two pseudoviruses tested for neutralization were produced with ES8 *env* genes amplified from a plasma that predated the test plasma by 3 months. Neutralization of these viruses was nearly identical to the neutralization of pseudoviruses carrying contemporaneous plasma Env. One pseudovirus tested for neutralization was produced with ES9 *env* amplified from a plasma isolated 5 months after the test plasma. This virus was neutralized less than some other autologous variants but more than one plasma virus that was contemporaneous with the test plasma. Taken together, these results suggest that in ES, titers of NAb against autologous, contemporaneous plasma viruses are low to undetectable, indicating that NAb do not play a direct role in viral suppression. Although some evidence for NAb escape was detected in ES6 and ES4, titers of NAb against archived proviral variants and noncontemporaneous plasma variants in other ES were low. This suggests that, in many cases, the lack of NAb targeting contemporaneous plasma virus is probably due to generally weak neutralizing antibody responses rather than viral escape from NAb. Thus, high titers of NAb against autologous viruses are apparently not required for the maintenance of viral suppression in most ES.

NAb against autologous virus does not play a dominant role in selecting plasma virus variants in HAART-treated patients. Autologous neutralization varied greatly between HAART-treated individuals and also between viral variants within individual patients (Fig. 5C and Table 2). One or more viral variants in all nine HAART-treated patients were neutralized <50% at a 1:20 plasma dilution. As shown in Fig. 5C, three patients, H26, H135, and H22, showed very little neutralization of either contemporaneous plasma viruses or proviral variants. No variants from H26 showed detectable neutralization at a 1:20 plasma titer. Only one of eight variants from H135 and one of eight variants from H22 were neutralized >50% at a 1:20 dilution of plasma. The remaining patients showed clearly detectable neutralization of some viral variants and very little neutralization of others. H28, H25, H154, and H9 showed maximum neutralization of autologous proviral variants, with IC_{50}s of 1:286, 1:164, 1:303, and 1:115, respectively. On the other hand, some viral variants from each of these patients were not detectably neutralized at a 1:20 plasma dilution. For H28 and H25, the least neutralized variants were plasma viruses. For H154, a plasma virus *env* variant that was amplified in five independent RT-PCRs was neutralized to a greater extent than some proviral isolates and a plasma virus *env* variant that was detected in only one RT-PCR. For patient H9, a plasma virus *env* variant that was amplified in three independent RT-PCRs was neutralized to a greater extent than mul-

TABLE 2. Neutralization of pseudoviruses by autologous plasma

Patient category and no.	Neutralization of plasma viruses[a]			Neutralization of proviruses[a]		
	Highest IC_{50}	Lowest IC_{50}	Median IC_{50}	Highest IC_{50}	Lowest IC_{50}	Median IC_{50}
Chronic progressors						
C61	0.0252	0.0110	0.0174	ND	ND	ND
C62	0.0126	0.0066	0.0102	ND	ND	ND
C93	0.0177	0.0079	0.0099	ND	ND	ND
C94	0.0500	0.0451	0.0500	ND	ND	ND
C96	0.0157	0.0012	0.0053	ND	ND	ND
C98	0.0500	0.0500	0.0500	ND	ND	ND
C109	0.0500	0.0100	0.0416	ND	ND	ND
Elite suppressors						
ES2	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
ES3	0.0319	0.0117	0.0218	0.0231[b]	0.0231[b]	0.0231[b]
ES4	0.0243	0.0104	0.0203	0.0049[b]	0.0049[b]	0.0049[b]
ES5	ND	ND	ND	0.0197[b]	0.0197[b]	0.0197[b]
ES6	0.0084[b]	0.0084[b]	0.0084[b]	0.0084	0.0020	0.0052
ES7	0.0500	0.0107	0.0304	0.0500	0.0500	0.0500
ES8	0.0110	0.0069	0.0101	0.0061[b]	0.0061[b]	0.0061[b]
ES9	0.0500	0.0058	0.0225	0.0093	0.0022	0.0058
ES10	0.0500	0.0114	0.0347	ND	ND	ND
HAART-treated patients						
H9	0.0109[b]	0.0109[b]	0.0109[b]	0.0500	0.0087	0.0211
H22	0.0500	0.0226	0.0500	0.0500	0.0500	0.0500
H23	0.0500	0.0335	0.0500	0.0500	0.0018	0.0155
H25	0.0500	0.0090	0.0500	0.0061[b]	0.0061[b]	0.0061[b]
H26	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
H28	0.0500	0.0500	0.0500	0.0186	0.0035	0.0127
H135	0.0500	0.0500	0.0500	0.0500	0.0439	0.0500
H148	0.0198	0.0169	0.0184	0.0500	0.0058	0.0096
H154	0.0500	0.0079	0.0289	0.0500	0.0033	0.0324

[a] The IC_{50} was calculated as the dilution of patient plasma at which infection of target cells by pseudovirus with autologous Env was 50% of the level measured in wells with control plasma. Pseudoviruses that were neutralized ≤50% at a 1:20 (0.05) plasma dilution were assigned an IC_{50} of 0.05 for purposes of statistical analysis. ND, not done.

[b] One representative *env* variant was tested for neutralization.

tiple proviral *env* variants. Some plasma virus variants in H25, H23, and H148 were also weakly neutralized by contemporaneous plasma. Taken together, these results suggest that titers of NAb against autologous virus are generally low in HAART-treated patients, and NAb does not appear to play a dominant role in selecting the viral variants that circulate in plasma in most individuals.

No correlation was found between the number of N-linked glycosylation sites or the V1-V5 length in *env* and the relative sensitivities of individual *env* clones to autologous NAb (not shown). Multiple factors in addition to these variables may influence the sensitivity to neutralization by strain-specific antibodies. In addition to many amino acid differences between variants, the locations of N-linked glycosylation sites were different between neutralization-sensitive and -resistant *env* clones (not shown). Subtle amino acid changes (19, 58, 64) and shifting glycosylation patterns (59) have each been shown to be sufficient for neutralizing antibody escape in viremic HIV-infected individuals.

Overall NAb titers against autologous virus did not differ significantly between chronic progressors, ES, and HAART-treated patients. In order to compare autologous neutralization titers between patient groups, we calculated a geometric mean autologous IC_{50} neutralization titer for plasma virus variants and proviral variants for each study subject (Fig. 6). The ranges and medians of these values were very similar for autologous neutralization of plasma viruses by chronic progressors and ES. Although many HAART-treated patients showed no neutralization of most plasma viral variants, the mean neutralization titers against autologous plasma viruses in these patients were also not significantly different from neutralization titers in chronic progressors or ES. ES showed a trend toward better neutralization of autologous proviral variants than autologous plasma virus variants (median IC_{50} titer, 1:108 versus 1:53), but this difference was also not statistically significant. Taken together, these results indicate that despite significant differences in viral diversity, numbers of N-linked glycans, V1-V5 lengths, anti-HIV binding antibody titers, and NAb titers against lab strains, titers of NAb against autologous virus did not differ significantly between chronic progressors, ES, and HAART-treated patients.

DISCUSSION

We examined the role of NAb in the control of viral replication in two populations of patients with undetectable viral loads, namely, ES and patients on HAART. *env* genes from ES have low diversity, few predicted N-linked glycans, and short V1-V5 lengths relative to *env* genes from chronic progressors and HAART-treated patients. Both ES and HAART-treated patients have lower titers of HIV-1 binding antibodies and

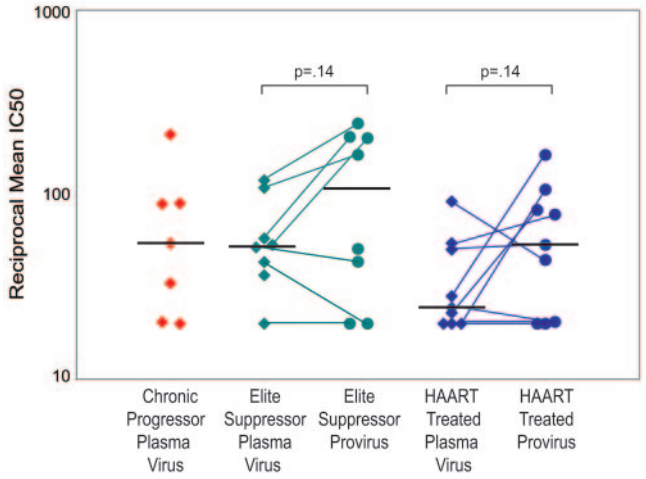

FIG. 6. Comparison between study groups of geometric mean reciprocal NAb titers against autologous virus. Each point represents the geometric mean reciprocal IC_{50} titer in plasma from an individual patient against all autologous plasma virus or proviral *env* variants tested from that patient. Horizontal hash marks indicate the median of the reciprocal IC_{50} values for each patient group. Mean reciprocal IC_{50} titers against pseudoviruses with plasma virus *env* genes and pseudoviruses with proviral *env* genes from the same subject are linked by lines.

lower titers of NAb against SF162 and NL4-3 than chronic progressors. Although both ES and HAART-treated patients showed significant neutralization of some autologous viral variants, neither group had significantly higher overall titers of NAb against autologous, contemporaneous plasma virus than chronic progressors.

The finding that ES have relatively low *env* diversity in both plasma viruses and cellular proviruses is in agreement with a previous study showing low diversity in proviruses from an ES (57). Since HIV-1 diversity increases over the course of a typical infection (52), this result suggests that extensive viral replication may never have occurred in these ES. These results are supported by recent work showing that some HIV-1-infected individuals who are HLA-B*57$^+$ suppress HIV-1 replication very early after infection (2). The HLA-B*57 allele is overrepresented in ES (37) and was present in seven of nine of the ES in this study. Low *env* diversity theoretically favors viral suppression by NAb in these subjects (25, 56). Although the low levels of replication occurring in these patients may have limited viral diversification, low antigen levels may also have prevented the stimulation of a high-titer antibody response. Thus, binding antibody titers as well as anti-SF162 and anti-NL4-3 NAb titers were low in these individuals. The low viral diversity seen in ES may be the result of rapid suppression of viremia, possibly by CD8$^+$ T cells, and may also be a critical factor in the maintenance of viral suppression. In addition to providing less opportunity for selection of NAb escape mutants, lower diversity may also provide less opportunity for selection of CTL escape mutants (20). It is thus possible that the rapid suppression of HIV-1 prior to extensive viral diversification is critical for long-term suppression of the virus by CD8$^+$ T cells.

We also found that *env* genes from ES have significantly shorter variable loops with less N-linked glycosylation than *env* genes from chronic progressors or HAART-treated patients. This finding is of interest given recent reports that relatively neutralization-sensitive viruses with short variable loops and low levels of glycosylation may predominate early after heterosexual transmission of HIV-1 subtypes C and A (9, 14). This was not found to be the case following homosexual transmission of subtype B virus (9, 18). Whether or not ES were initially infected with viruses with short variable loops and low levels of glycosylation, viruses with these characteristics have become predominant in these subjects, as they are present in plasma as well as archived in resting CD4$^+$ T cells. These findings suggest that antibody pressure in these individuals may have never reached levels sufficient to drive the selection of *env* variants with long variable loops and extensive glycosylation.

env genes from HAART-treated patients had sequence diversity, variable loop lengths, and levels of N-linked glycosylation similar to those of *env* genes from chronic progressors. This is reasonable given that these patients had high levels of viral replication for years prior to HAART treatment. During that time, viruses in these patients most likely experienced similar levels of antibody pressure to those seen in chronic progressors. The maintenance of viral diversity and *env* characteristics similar to those in viremic patients can most likely be attributed to stable archiving of latent virus in the resting CD4$^+$ T-cell reservoir. Random reactivation and release of this virus could explain the maintenance of diversity, glycosylation, and variable loop length in plasma virus despite low antibody titers (22, 54).

We found that both ES and HAART-treated patients had significantly lower anti-HIV-1 binding antibody titers than chronic progressors. Since ES have high CD4$^+$ T-cell counts and relatively normal immune responses, the low antibody titers in these individuals are not the result of immunodeficiency. It is more likely that they are the result of extremely low levels of antigenic stimulation. Low antigen levels likely play a role in the low binding antibody titers seen in HAART-treated patients as well. It is also possible that some loss of antibody response in HAART patients is the result of CD4$^+$ T-cell depletion and a loss of T-cell help prior to HAART treatment.

We also examined titers of NAb against well-characterized subtype B reference strains and autologous viruses. We found that NAb titers against the lab strain SF162 were significantly lower in ES and HAART-treated patients than in chronic progressors. While the majority of chronic progressors showed detectable neutralization of the reference strain NL4-3, no ES or HAART-treated patients showed neutralizing antibody activity against this virus. These results are in agreement with previous studies showing that SF162 is more neutralization sensitive than NL4-3 (35). The lack of detectable NAb against NL4-3 in ES and HAART-treated patients despite detectable NAb titers against SF162 might also indicate that NAb in these two patient groups target different epitopes than NAb in chronic progressors. Overall, these experiments served as a screen for heterologous or broadly neutralizing antibodies (35). Several previous studies of LTNPs with higher viral loads concluded that these patients had relatively high titers of heterologous neutralizing antibody (7, 39, 42, 45). This is clearly not the case for ES. Viral diversity is similar in HAART-treated patients and chronic progressors, suggesting that pressure that could have driven the generation of broadly neutral-

izing antibodies was present in HAART-treated individuals. Thus, the low titers of heterologous neutralizing antibody in HAART-treated patients despite a relatively high viral diversity suggest that high levels of antigenic stimulation may be necessary to maintain cross-reactive NAb against HIV-1 lab strains (35, 40, 46).

Although binding Ab and heterologous NAb titers were low in ES and HAART-treated patients, these findings did not rule out the presence of high-titer, strain-specific antibodies against autologous viruses. Since neutralization by contemporaneous plasma recapitulates the in vivo interaction between viruses and NAb at the time of sampling, we tested the neutralization of plasma virus by autologous contemporaneous plasma. We found that despite the low diversity, low levels of glycosylation, and short V1-V5 lengths of plasma virus Env in ES, these subjects did not have high-titer NAb against autologous viruses. In fact, neutralization of several plasma virus variants was undetectable at the plasma concentrations tested. Although some proviral variants were relatively well neutralized by autologous plasma, overall autologous neutralization in ES did not surpass that seen in chronic progressors. It is noteworthy that despite the fact that ES have significantly lower anti-HIV binding antibody titers and anti-SF162 NAb titers than chronic progressors, ES and chronic progressors have similar titers of NAb against autologous, contemporaneous plasma viruses. This may indicate that the immune response in ES is more effective in developing NAb than the response in chronic progressors. However, HAART-treated patients also have significantly lower binding antibody and anti-SF162 NAb titers than chronic progressors. Like ES, their overall NAb titers against autologous, contemporaneous viruses are similar to those detected in chronic progressors. This suggests that these characteristics are not particular to the antibody response in ES. Thus, titers of NAb against autologous, contemporaneous plasma viruses are low in ES, and antibody responses in ES do not appear to be generally more effective at neutralization than those detected in HAART-treated patients. Taken together, these observations indicate that NAb most likely do not play a dominant role in the maintenance of viral suppression in ES.

These findings are supported by a recent study of simian-human immunodeficiency virus suppression in Mamu-A*01-positive macaques. This study showed that suppression of an attenuated strain of simian-human immunodeficiency virus was maintained in Mamu-A*01-positive macaques despite the depletion of B cells (34). Interestingly, the depletion of B cells from Mamu-A*01-negative macaques in the same study resulted in a loss of viral suppression. B cells may therefore play a role in lentiviral suppression, but this role may be less important in animals or humans with particularly robust $CD8^+$ T-cell responses and effective viral suppression.

In addition to high viral diversity, HAART-treated patients also exhibited significant variation in NAb titers against autologous viruses. Some patients showed almost no autologous virus neutralization. Others showed relatively high titers of NAb against some viral variants and almost no neutralization of others. It may be that the viral diversity is too high and the antigenic stimulation is too low for significant NAb responses to be maintained against all viral variants. Interestingly, contemporaneous plasma virus variants in several patients were neutralized more strongly than proviral isolates that were not found in the plasma. For two patients, plasma virus variants with significantly different sensitivities to autologous neutralization coexisted in the plasma. This may mean that the NAb titers observed in these patients are not high enough to exert strong selective pressure on HIV-1; it may also be a result of the manner in which plasma viruses are produced in patients on HAART. Since plasma viruses in these patients may be the result of random release of archived viruses rather than ongoing replication, the effects of weak negative selection may be much more difficult to detect than they would be in viremic patients (22).

In summary, we have found that *env* genes in ES have surprisingly low diversity, few predicted N-linked glycans, and short V1-V5 lengths. These values are similar, however, between *env* genes from chronic progressors and those from HAART-treated patients. Both ES and HAART-treated patients have lower titers of HIV-1 binding antibodies, NAb against SF162, and NAb against NL4-3 than chronic progressors. Although both ES and HAART-treated patients showed significant neutralization of some autologous viral variants, neither group had significantly higher overall titers of NAb against autologous, contemporaneous plasma viruses than chronic progressors. Titers of NAb against plasma viruses were also not significantly higher than titers against proviral variants. Thus, high-titer neutralizing antibodies against heterologous virus or autologous, contemporaneous plasma virus are not required for the maintenance of suppression in ES, and they appear to play only a minor role in selecting plasma virus variants in patients on suppressive HAART. Both drug-mediated and natural suppression of HIV-1 replication to levels below 50 copies/ml may limit the stimulation and maintenance of effective NAb responses.

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REFERENCES

1. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo.** 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS **4:**107–112.
2. **Altfeld, M., M. M. Addo, E. S. Rosenberg, F. M. Hecht, P. K. Lee, M. Vogel, X. G. Yu, R. Draenert, M. N. Johnston, D. Strick, T. M. Allen, M. E. Feeney, J. O. Kahn, R. P. Sekaly, J. A. Levy, J. K. Rockstroh, P. J. Goulder, and B. D. Walker.** 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS **17:**2581–2591.
3. **Binley, J. M., A. Trkola, T. Ketas, D. Schiller, B. Clas, S. Little, D. Richman, A. Hurley, M. Markowitz, and J. P. Moore.** 2000. The effect of highly active antiretroviral therapy on binding and neutralizing antibody responses to human immunodeficiency virus type 1 infection. J. Infect. Dis. **182:**945–949.
4. **Bradney, A. P., S. Scheer, J. M. Crawford, S. P. Buchbinder, and D. C. Montefiori.** 1999. Neutralization escape in human immunodeficiency virus type 1-infected long-term nonprogressors. J. Infect. Dis. **179:**1264–1267.
5. **Burns, D. P., C. Collignon, and R. C. Desrosiers.** 1993. Simian immunodeficiency virus mutants resistant to serum neutralization arise during persistent infection of rhesus monkeys. J. Virol. **67:**4104–4113.
6. **Burton, D. R.** 2002. Antibodies, viruses and vaccines. Nat. Rev. Immunol. **2:**706–713.
7. **Cao, Y., L. Qin, L. Zhang, J. Safrit, and D. D. Ho.** 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N. Engl. J. Med. **332:**201–208.
8. **Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison.** 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature **433:**834–841.
9. **Chohan, B., D. Lang, M. Sagar, B. Korber, L. Lavreys, B. Richardson, and**

J. Overbaugh. 2005. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J. Virol. **79:**6528–6531.
10. **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387:**183–188.
11. **Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci.** 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc. Natl. Acad. Sci. USA **95:**8869–8873.
12. **Chun, T. W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R. F. Siliciano.** 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat. Med. **1:**1284–1290.
13. **Decker, J. M., F. Bibollet-Ruche, X. Wei, S. Wang, D. N. Levy, W. Wang, E. Delaporte, M. Peeters, C. A. Derdeyn, S. Allen, E. Hunter, M. S. Saag, J. A. Hoxie, B. H. Hahn, P. D. Kwong, J. E. Robinson, and G. M. Shaw.** 2005. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. J. Exp. Med. **201:**1407–1419.
14. **Derdeyn, C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen, and E. Hunter.** 2004. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science **303:**2019–2022.
15. **Dornadula, G., H. Zhang, B. VanUitert, J. Stern, L. Livornese, Jr., M. J. Ingerman, J. Witek, R. J. Kedanis, J. Natkin, J. DeSimone, and R. J. Pomerantz.** 1999. Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. JAMA **282:**1627–1632.
16. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278:**1295–1300.
17. **Frenkel, L. M., Y. Wang, G. H. Learn, J. L. McKernan, G. M. Ellis, K. M. Mohan, S. E. Holte, S. M. De Vange, D. M. Pawluk, A. J. Melvin, P. F. Lewis, L. M. Heath, I. A. Beck, M. Mahalanabis, W. E. Naugler, N. H. Tobin, and J. I. Mullins.** 2003. Multiple viral genetic analyses detect low-level human immunodeficiency virus type 1 replication during effective highly active antiretroviral therapy. J. Virol. **77:**5721–5730.
18. **Frost, S. D., Y. Liu, S. L. Pond, C. Chappey, T. Wrin, C. J. Petropoulos, S. J. Little, and D. D. Richman.** 2005. Characterization of human immunodeficiency virus type 1 (HIV-1) envelope variation and neutralizing antibody responses during transmission of HIV-1 subtype B. J. Virol. **79:**6523–6527.
19. **Frost, S. D., T. Wrin, D. M. Smith, S. L. Pond, Y. Liu, E. Paxinos, C. Chappey, J. Galovich, J. Beauchaine, C. J. Petropoulos, S. J. Little, and D. D. Richman.** 2005. Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. Proc. Natl. Acad. Sci. USA **102:**18514–18519.
20. **Goulder, P. J., and D. I. Watkins.** 2004. HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. **4:**630–640.
21. **Harrer, T., E. Harrer, S. A. Kalams, T. Elbeik, S. I. Staprans, M. B. Feinberg, Y. Cao, D. D. Ho, T. Yilma, A. M. Caliendo, R. P. Johnson, S. P. Buchbinder, and B. D. Walker.** 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. AIDS Res. Hum. Retrovir. **12:**585–592.
22. **Hermankova, M., S. C. Ray, C. Ruff, M. Powell-Davis, R. Ingersoll, R. T. D'Aquila, T. C. Quinn, J. D. Siliciano, R. F. Siliciano, and D. Persaud.** 2001. HIV-1 drug resistance profiles in children and adults with viral load of <50 copies/ml receiving combination therapy. JAMA **286:**196–207.
23. **Javaherian, K., A. J. Langlois, G. J. LaRosa, A. T. Profy, D. P. Bolognesi, W. C. Herlihy, S. D. Putney, and T. J. Matthews.** 1990. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. Science **250:**1590–1593.
24. **Johnson, W. E., and R. C. Desrosiers.** 2002. Viral persistence: HIV's strategies of immune system evasion. Annu. Rev. Med. **53:**499–518.
25. **Joos, B., A. Trkola, M. Fischer, H. Kuster, P. Rusert, C. Leemann, J. Boni, A. Oxenius, D. A. Price, R. E. Phillips, J. K. Wong, B. Hirschel, R. Weber, and H. F. Gunthard.** 2005. Low human immunodeficiency virus envelope diversity correlates with low in vitro replication capacity and predicts spontaneous control of plasma viremia after treatment interruptions. J. Virol. **79:**9026–9037.
26. **Kieffer, T. L., P. Kwon, R. E. Nettles, Y. Han, S. C. Ray, and R. F. Siliciano.** 2005. G→A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting $CD4^+$ T cells in vivo. J. Virol. **79:**1975–1980.
27. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
28. **Kimura, T., K. Yoshimura, K. Nishihara, Y. Maeda, S. Matsumi, A. Koito, and S. Matsushita.** 2002. Reconstitution of spontaneous neutralizing antibody response against autologous human immunodeficiency virus during highly active antiretroviral therapy. J. Infect. Dis. **185:**53–60.
29. **Kwong, P. D., M. L. Doyle, D. J. Casper, C. Cicala, S. A. Leavitt, S. Majeed, T. D. Steenbeke, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P. W. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt, J. Sodroski, W. A. Hendrickson, and J. Arthos.** 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature **420:**678–682.
30. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659.
31. **Li, M., F. Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori.** 2005. Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125.
32. **Liu, S. L., A. G. Rodrigo, R. Shankarappa, G. H. Learn, L. Hsu, O. Davidov, L. P. Zhao, and J. I. Mullins.** 1996. HIV quasispecies and resampling. Science **273:**415–416.
33. **Lu, M., S. C. Blacklow, and P. S. Kim.** 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat. Struct. Biol. **2:**1075–1082.
34. **Mao, H., B. A. Lafont, T. Igarashi, Y. Nishimura, C. Brown, V. Hirsch, A. Buckler-White, R. Sadjadpour, and M. A. Martin.** 2005. $CD8^+$ and $CD20^+$ lymphocytes cooperate to control acute simian immunodeficiency virus/human immunodeficiency virus chimeric virus infections in rhesus monkeys: modulation by major histocompatibility complex genotype. J. Virol. **79:**14887–14898.
35. **Mascola, J. R., P. D'Souza, P. Gilbert, B. H. Hahn, N. L. Haigwood, L. Morris, C. J. Petropoulos, V. R. Polonis, M. Sarzotti, and D. C. Montefiori.** 2005. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. J. Virol. **79:**10103–10107.
36. **McKeating, J. A., J. Gow, J. Goudsmit, L. H. Pearl, C. Mulder, and R. A. Weiss.** 1989. Characterization of HIV-1 neutralization escape mutants. AIDS **3:**777–784.
37. **Migueles, S. A., M. S. Sabbaghian, W. L. Shupert, M. P. Bettinotti, F. M. Marincola, L. Martino, C. W. Hallahan, S. M. Selig, D. Schwartz, J. Sullivan, and M. Connors.** 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc. Natl. Acad. Sci. USA **97:**2709–2714.
38. **Monie, D., R. P. Simmons, R. E. Nettles, T. L. Kieffer, Y. Zhou, H. Zhang, S. Karmon, R. Ingersoll, K. Chadwick, H. Zhang, J. B. Margolick, T. C. Quinn, S. C. Ray, M. Wind-Rotolo, M. Miller, D. Persaud, and R. F. Siliciano.** 2005. A novel assay allows genotyping of the latent reservoir for human immunodeficiency virus type 1 in the resting $CD4^+$ T cells of viremic patients. J. Virol. **79:**5185–5202.
39. **Montefiori, D. C., G. Pantaleo, L. M. Fink, J. T. Zhou, J. Y. Zhou, M. Bilska, G. D. Miralles, and A. S. Fauci.** 1996. Neutralizing and infection-enhancing antibody responses to human immunodeficiency virus type 1 in long-term nonprogressors. J. Infect. Dis. **173:**60–67.
40. **Moore, J. P., Y. Cao, L. Qing, Q. J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C. F. Barbas III, D. R. Burton, and D. D. Ho.** 1995. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. **69:**101–109.
41. **Nettles, R. E., T. L. Kieffer, P. Kwon, D. Monie, Y. Han, T. Parsons, J. Cofrancesco, Jr., J. E. Gallant, T. C. Quinn, B. Jackson, C. Flexner, K. Carson, S. Ray, D. Persaud, and R. F. Siliciano.** 2005. Intermittent HIV-1 viremia (blips) and drug resistance in patients receiving HAART. JAMA **293:**817–829.
42. **Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, L. K. Schrager, et al.** 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N. Engl. J. Med. **332:**209–216.
43. **Pierson, T., T. L. Hoffman, J. Blankson, D. Finzi, K. Chadwick, J. B. Margolick, C. Buck, J. D. Siliciano, R. W. Doms, and R. F. Siliciano.** 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J. Virol. **74:**7824–7833.
44. **Pierson, T. C., Y. Zhou, T. L. Kieffer, C. T. Ruff, C. Buck, and R. F. Siliciano.** 2002. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. J. Virol. **76:**8518–8531.
45. **Pilgrim, A. K., G. Pantaleo, O. J. Cohen, L. M. Fink, J. Y. Zhou, J. T. Zhou, D. P. Bolognesi, A. S. Fauci, and D. C. Montefiori.** 1997. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. J. Infect. Dis. **176:**924–932.
46. **Pinter, A., W. J. Honnen, Y. He, M. K. Gorny, S. Zolla-Pazner, and S. C. Kayman.** 2004. The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. J. Virol. **78:**5205–5215.

47. **Poignard, P., R. Sabbe, G. R. Picchio, M. Wang, R. J. Gulizia, H. Katinger, P. W. Parren, D. E. Mosier, and D. R. Burton.** 1999. Neutralizing antibodies have limited effects on the control of established HIV-1 infection in vivo. Immunity **10:**431–438.
48. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. **4:**679–684.
49. **Reitz, M. S., Jr., C. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff.** 1988. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell **54:**57–63.
50. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. USA **100:**4144–4149.
51. **Ruff, C. T., S. C. Ray, P. Kwon, R. Zinn, A. Pendleton, N. Hutton, R. Ashworth, S. Gange, T. C. Quinn, R. F. Siliciano, and D. Persaud.** 2002. Persistence of wild-type virus and lack of temporal structure in the latent reservoir for human immunodeficiency virus type 1 in pediatric patients with extensive antiretroviral exposure. J. Virol. **76:**9481–9492.
52. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I. Mullins.** 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. **73:**10489–10502.
53. **Skrabal, K., S. Saragosti, J. L. Labernardiere, F. Barin, F. Clavel, and F. Mammano.** 2005. Human immunodeficiency virus type 1 variants isolated from single plasma samples display a wide spectrum of neutralization sensitivity. J. Virol. **79:**11848–11857.
54. **Tobin, N. H., G. H. Learn, S. E. Holte, Y. Wang, A. J. Melvin, J. L. McKernan, D. M. Pawluk, K. M. Mohan, P. F. Lewis, J. I. Mullins, and L. M. Frenkel.** 2005. Evidence that low-level viremias during effective highly active antiretroviral therapy result from two processes: expression of archival virus and replication of virus. J. Virol. **79:**9625–9634.
55. **Tremblay, M., and M. A. Wainberg.** 1990. Neutralization of multiple HIV-1 isolates from a single subject by autologous sequential sera. J. Infect. Dis. **162:**735–737.
56. **Trkola, A., H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto, and H. F. Gunthard.** 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat. Med. **11:**615–622.
57. **Wang, B., M. Mikhail, W. B. Dyer, J. J. Zaunders, A. D. Kelleher, and N. K. Saksena.** 2003. First demonstration of a lack of viral sequence evolution in a nonprogressor, defining replication-incompetent HIV-1 infection. Virology **312:**135–150.
58. **Watkins, B. A., S. Buge, K. Aldrich, A. E. Davis, J. Robinson, M. S. Reitz, Jr., and M. Robert-Guroff.** 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. J. Virol. **70:**8431–8437.
59. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
60. **Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman.** 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278:**1291–1295.
61. **Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705–711.
62. **Yoshiyama, H., H. Mo, J. P. Moore, and D. D. Ho.** 1994. Characterization of mutants of human immunodeficiency virus type 1 that have escaped neutralization by a monoclonal antibody to the gp120 V2 loop. J. Virol. **68:**974–978.
63. **Zhang, H., Y. Zhou, C. Alcock, T. Kiefer, D. Monie, J. Siliciano, Q. Li, P. Pham, J. Cofrancesco, D. Persaud, and R. F. Siliciano.** 2004. Novel single-cell-level phenotypic assay for residual drug susceptibility and reduced replication capacity of drug-resistant human immunodeficiency virus type 1. J. Virol. **78:**1718–1729.
64. **Zhang, P. F., P. Bouma, E. J. Park, J. B. Margolick, J. E. Robinson, S. Zolla-Pazner, M. N. Flora, and G. V. Quinnan, Jr.** 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J. Virol. **76:**644–655.