Epitopes Immediately Below the Base of the V3 Loop of gp120 as Targets for the Initial Autologous Neutralizing Antibody Response in Two HIV-1 Subtype B-Infected Individuals

Running Title: Autologous neutralization epitopes on HIV-1

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ABSTRACT

Epitopes that drive the initial autologous neutralizing antibody response in HIV-1-infected individuals could provide insights for vaccine design. Though highly strain-specific, these epitopes are immunogenic, vulnerable to antibody attack on infectious virus and could be involved in the ontogeny of broadly neutralizing antibody responses. To delineate such epitopes, we used site-directed mutagenesis, autologous plasma samples and autologous monoclonal antibodies to map the amino acid changes that led to escape from the initial autologous neutralizing antibody response in two HIV-1 subtype B-infected individuals. Additional mapping of the epitopes was accomplished by using alanine scanning mutagenesis. Escape in the two individuals occurred by different pathways but the response in both cases appeared to be directed against the same region of gp120. In total, three amino acid positions were identified that were independently associated with autologous neutralization. Positions 295 and 332 are located immediately before and after the N- and C-terminal cysteines of the V3 loop, respectively, the latter of which affected an N-linked glycan that was critical to the neutralization epitope. Position 415 affected an N-linked glycan at position 413 in the C-terminus of V4 that might mask epitopes near the base of V3. All three sites lie in close proximity on a four-stranded anti-parallel sheet on the outer domain of gp120. We conclude that a region just below the base of the V3 loop, near the coreceptor binding domain of gp120, can be a target for autologous neutralization.
INTRODUCTION

Infection with HIV-1 is accompanied by a potent autologous neutralizing antibody (nAb) response that for the first 1-2 years of infection is relatively specific for the initial virus variant that arises in peripheral circulation (9, 16, 31, 32, 46, 60, 64, 73, 74, 77). The nAb response broadens in subsequent years, with an estimated 10-20% of chronically infected subjects exhibiting potent neutralizing activity against a broad spectrum of genetic variants (5, 22, 32, 49, 51, 60, 87, 89, 91). Little is known about the requirements to elicit broadly nAb responses with vaccines (92).

The autologous nAb response develops slowly, usually becoming detectable after several months of infection (9, 16, 23, 64, 73, 74, 77), and is accompanied by the emergence of escape variants that dominate earlier variants (31, 100). Over time in an infected individual, repeated cycles of autologous virus neutralization and escape have been observed (1, 2, 7, 8, 27, 62, 77, 100). The ability of HIV-1 to escape nAbs so efficiently and repeatedly offers an explanation for why nAbs fail to control the virus during chronic infection. Despite this limitation, passive transfer experiments in nonhuman primates have demonstrated that pre-existing nAbs, including broadly neutralizing monoclonal antibodies (mAbs), can protect against the acquisition of AIDS virus infection after intravenous and mucosal challenge (25, 33, 34, 55, 59, 72, 90).

Neutralizing Abs target the surface gp120 and transmembrane gp41 envelope (Env) glycoproteins that assemble into trimolecular complexes of gp120-gp41 heterodimers on the virus surface to mediate virus entry into host cells (13, 14, 102). Sequence variability and epitope masking by N-linked glycans and other structural constraints in the context of functional Env glycoprotein spikes contribute to a general nAb evasion strategy that poses difficult challenges for vaccine development (41,
71, 100, 102). Nonetheless, nAb epitopes do exist that are both conserved and exposed; these are best illustrated by the broadly neutralizing activity of a small number of human mAbs against gp120 (10, 18, 56, 96, 98, 103) and gp41 (56, 68, 93, 107). These conserved epitopes are of major interest for vaccine research but so far their potential has been severely limited by poor immunogenicity (36, 58).

The initial autologous nAb response has received little attention for vaccine development because of its limited breadth. Nonetheless, the immunogenicity and vulnerability of these epitopes are quite striking. Moreover, it is not known whether these epitopes are involved in the ontogeny of broadly nAb responses. Epitopes for autologous nAbs can be identified by comparing the Env sequences of early neutralization-sensitive viruses and later escape variants to identify possible escape mutations. Because some mutations could arise for other reasons, including pressure from other immune responses, additional experiments are needed to identify specific mutations that contributed to escape. Such detailed genetic analyses are complicated by the use of polyclonal antisera and by the discontinuous, conformational nature of many epitopes. Also, some epitopes could be subject to allosteric influences originating from regions outside the antibody contact site. To partially overcome these obstacles, several recent studies have used mAbs and chimeric and site-directed mutant Envs for epitope mapping experiments. These studies have mostly focused on subtype C infections and have shown that early autologous nAbs can target multiple epitopes in the V1V2, C3-V4 and V5 regions of gp120 (54, 65-67, 80-82). These studies also show that escape can occur through multiple pathways, including substitutions, deletions, insertions and the addition of N-linked glycans.

Much less is known about the specificity of the initial autologous nAb response in non-subtype C infection. Studies of mutational patterns and Env structure have indicated a degree of subtype-preference in neutralization epitopes and escape pathways (28). Although subtype B and C infections
share common pathways for escape (8, 26, 30, 54, 85, 100), detailed genetic studies of the epitopes in subtype B infection are lacking. Wei et al. (100) used site-directed mutagenesis to demonstrate that the simultaneous addition of N-linked glycans at positions 240 (C2), 268 (C2), 301 (V3) and in V4 completely restored the escape phenotype in an early sensitive subtype B variant. In another subtype B infected subject in that study, escape involved the addition of N-linked glycans at positions 235 (C2), 359 (C3) and in V4. In a study of subtype B simian-human immunodeficiency virus (SHIV), autologous neutralization was mapped to the V1V2 region of gp120 (44).

We investigated the initial autologous nAb response and subsequent escape in two HIV-1 subtype B-infected individuals (AC10 and AC13) by using a combination of mAbs, site-directed mutagenesis and alanine scanning. We provide evidence for the presence of previously unknown autologous nAb epitopes located immediately below the base of the V3-loop, near the coreceptor binding site on gp120. Additional evidence suggests this region is susceptible to epitope masking by an N-linked glycan in the C-terminus of V4.

MATERIALS AND METHODS

Plasma samples and virus isolates. Plasma samples were collected at multiple time points as part of a supervised therapy interruption study (approved by the Partners AIDS Research Center human research committee) from two individuals (AC10 and AC13) who were diagnosed with acute HIV-1-infection and who initiated antiretroviral therapy (ART) soon thereafter (63, 83). Both subjects were plasma viral RNA positive and either ELISA negative (AC13) or weakly ELISA positive and Western blot positive for p18, p24 and gp160 (AC10) at the time of diagnosis in 1998. They interrupted ART after 1.5 years (AC10) and 1.0 years (AC13). AC10 remained off ART for the remainder of the study period. AC13 re-initiated ART on day 454 and discontinued therapy on day 566.
Virus was isolated by peripheral blood mononuclear cell (PBMC) coculture from both subjects (63). Virus from AC10 was isolated at the time of diagnosis (day 0, prior to ART) and again at day 119 post therapy interruption (1.9 years from diagnosis). This subject was weakly positive by Western blot (WB) during the last 73 days of therapy and became strongly WB positive within 21 days after therapy interruption (62). Virus from AC13 was isolated on days 134 and 237 after therapy interruption (1.35 and 1.63 years from enrollment, respectively). Both subjects were negative for autologous nAbs immediately after the first interruption of therapy (62, 63). Rev/env cassettes were cloned from PBMC DNA and inserted directly into pcDNA 3.1D/V5-His-TOPO (Invitrogen Corp., Carlsbad, CA) as described (47). Clones with full-length inserts in the correct orientation were co-transfected with an env-deficient HIV-1 backbone plasmid (pSG3Δenv) in 293T cells to produce Env-pseudotyped viruses. A single clone from each time point for both subjects was identified that was infectious in TZM-bl cells and used for further study.

Additional plasma samples were obtained from chronically infected individuals in the United States, Malawi, South Africa and Tanzania who were enrolled in the Center for HIV/AIDS Vaccine Immunology (CHAVI) protocols 001 and 008. Additional Env-pseudotyped viruses were obtained from individuals who were infected with HIV-1 subtype B (QH0692.42, SC422661.8, PVO.4, TRO.11, RHPA4259.7, THRO4156.18, REJO4541.67, TRJO4551.58, WITO4160.33, CAAN5342.A2) (47), subtype C (Du156.12, Du172.17, Du422.1, ZM197M.PB7, ZM214M.PL15, ZM233M.PB6, ZM249M.PL1, ZM53M.PB12, ZM109F.PB4, ZM135M.PL10a, CAP45.2.00.G3, CAP210.2.00.E8) (50) and subtype A (Q23.17, Q168.a2, Q461.e2, Q259.d2.17) (53, 76). The majority of these viruses are from acute and early HIV-1 infection. An additional set of viruses was obtained from individuals who were chronically infected with HIV-1 subtype B in Lima, Peru (H022.7, H029.12, H030.7, H031.7, H035.18, H061.14,
Isolation mAbs from AC10 and AC13. Viably frozen PBMC were obtained from subjects AC10 and AC13 after 1.75 and 2.23 years from diagnosis, respectively. Autologous nAbs were present in both individuals at these time points. B cells were inoculated with EBV (B95-8 strain) and plated at low cell densities in multiple 96-well tissue culture plates containing irradiated, mature human macrophage feeder cells prepared from HIV-1-seronegative subjects as previously described (104). After the second week of culture, all wells contained proliferating oligoclonal transformed B cells, at which time culture fluids were screened for antibody reactivity by ELISA (78, 104) and by neutralization assays with the corresponding early autologous virus. B cells in antibody positive wells were serially subcultured at decreasing cell numbers (78) to achieve outgrowth of clonal B cells. To improve growth and antibody production, cells producing mAbs LG1.1C and 5.9C were converted to hybridomas by fusion with the HAT-sensitive HMMA cells (75) as previously reported (104). The mAbs were purified from culture supernatants by protein A affinity chromatography. Although the B cell line producing mAb LG3.2A was lost due to low liquid nitrogen levels in a cryopreservation tank following Hurricane Katrina, a sufficient quantity of the mAb had been produced to allow the studies described in this report.

Genetic analysis of the variable regions of heavy- and light-chain (\(V_H\) and \(V_L\)) genes of mAb 5.9C. Genes encoding variable-heavy (\(V_H\)) and variable-light (\(V_L\)) regions were amplified by RT/PCR using a modified method as describe (52, 95). Briefly RNA isolated from mAb 5.9C producer cells was reverse transcribed using Superscript III in the presence of primers specific for human IgG, IgM, IgD, IgA1, IgA2, kappa and lambda constant gene regions (52). The \(V_H\), \(V_N\) and \(V_L\) genes were amplified from cDNAs separately in nested PCR as described (52). PCR products were purified and sequenced. The \(V_H\) and \(V_L\) Reven\(env\) cassettes of these latter viruses were derived from PBMC DNA after virus isolation by PBMC co-culture.
gene segments and the potential functionality of the Ig genes were determined using the SoDA program (97).

**Monoclonal Ab cross-competition analysis.** The mAbs LG1.1C and 5.9C were tested for cross-competition with each other as described previously (79) to determine whether they recognized overlapping sites on gp120. Non-competing human mAbs 1.7B and 1.4E were included as controls; 1.7B binds an epitope in the co-receptor binding region of gp120 (104), whereas 1.4E binds an epitope in the V3 loop of gp120 (unpublished). Briefly, 293T-derived AC10.d0 Env-pseudotyped virus was solubilized with Triton-X100 and the gp120 captured in wells of enzyme-linked immunosorbant (ELISA) plates (Corning) coated with human mAb A32, which binds gp120 near the CD4-binding site (101). HIV-1 Env glycoprotein prepared and captured this way binds gp120-specific but not gp41-specific antibodies (unpublished). The plates were washed and blocked with 4% whey/0.5% Tween 20 in phosphate buffered saline (PBS) for 30 minutes. Unlabeled human mAbs (10 µg/ml), or dilution buffer, was added to the wells and incubated at room temperature for 30 minutes. Without washing the plates, optimal dilutions of biotinylated human mAbs were added directly to the wells containing unlabeled competitor mAbs or buffer. After a wash step, peroxidase-conjugated streptavidin was incubated in the wells. Color was developed with TMB+H2O2 as substrate and the reaction was stopped with 1 M phosphoric acid. Color was read as the optical density (OD) at 450 nm. Percent binding was calculated as the OD observed with competitor mAb divided by the OD without competitor and multiplied by 100.

**Site-directed mutagenesis and alanine scanning.** Mutations were generated using the Quick-change mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA, USA). Primer sequences are available on request from the authors. The entire gp160 gene of each mutated plasmid was sequenced to verify that only the intended mutations had been introduced. Sequence
analysis was performed by cycle-sequencing and dye terminator methods with an automated DNA 
Sequenator (model 3100; Applied Biosystems, Inc.) as recommended by the manufacturer. Individual 
sequence fragments for each gp160 clone were assembled and edited using the Sequencher program 
4.2 (Gene Codes Corp., Ann Arbor, MI). Nucleotide and deduced amino acid sequences were initially 
aligned using CLUSTAL W (35, 94) and were manually adjusted for an optimal alignment in hypervariable 
domains by using MASE (24). All sequence locations reported here are based on the HXB2 numbering 
system.

**Neutralization assay.** Neutralizing Abs were measured as a function of reductions in luciferase 
reporter gene expression after a single round of infection in TZM-bl (also known as JC53-bl) cells as 
described (47, 61). This is a modified version of the assay as described previously (100). Briefly, 200 
TCID50 of pseudovirus was incubated with serial 3-fold dilutions of heat-inactivated plasma samples in 
duplicate or triplicate in a total volume of 150 μl for 1 hr at 37°C in 96-well flat-bottom culture plates. 
Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 75 μg/ml DEAE dextran) 
were added to each well. One set of control wells received cells + Env-pseudotyped virus (virus control) 
and another set received cells only (background control). After 48 hour incubation, 100 μl of cells was 
transferred to 96-well black solid plates (Costar) for measurements of luminescence using the Britelite 
Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences). The 50% inhibitory dose (ID50) 
was defined as the sample dilution that caused a 50% reduction in RLU compared to virus control wells 
after subtraction of background RLU. In the case of purified mAbs (LG1.1C and LG3.2A), 50% inhibitory 
concentration (IC50) was used. Assay stocks of Env-pseudotyped viruses were prepared by transfection 
in 293T cells and were titrated in TZM-bl cells as described (47, 61). To avoid artifacts due to residual 
plasma levels of antiretroviral drugs, all plasma samples used for nAb assays were obtained at time 
points when subjects were ART-free.
**Molecular modeling and molecular dynamics simulations.** All-atom molecular dynamics simulations were performed on the YU2 gp120 structure (1RZK) (42). Missing loops were modeled for clarity as described previously (6). The gp120 protein was solvated in 16,304 water molecules to provide a system with 55,130 atoms. Periodic boundary conditions were applied and the particle mesh Ewald approach was used to treat long-range electrostatic interactions (19). The temperature was maintained at 300 K, using the Berendsen temperature algorithm (4). A 2 fs time step was used and all bonds involving hydrogen atoms were constrained using the SHAKE algorithm (84). Simulations were carried out for approximately 16 ns and the last 10 ns were considered for analysis. Simulations were performed using the force field described by Cornell et al. (17) and the AMBER 6.0 suite of programs (12). Discussions of an unliganded gp120 core structure were based on the fully glycosylated simian immunodeficiency virus (SIV) X-ray structure (PDB code 2BF1) (15). Three-dimensional images were generated using VMD (38).

**RESULTS**

**Documentation of autologous neutralization and escape using clonal Envs from subjects AC10 and AC13.** Subjects AC10 and AC13 were participants in a study of treatment interruption in HIV-1-infected individuals who initiated ART during primary infection (83). They were chosen because they exhibited the strongest initial autologous nAb response among 9 subjects studied previously (63). Time points for plasma collection, virus isolation and mAb recovery are shown in Figure 1. AC10 discontinued therapy after 1.5 years and remained off therapy thereafter. Plasma viremia was below the level of detection for the last 16 months prior to the cessation of ART and rebounded to a peak of 5,610 copies/ml 40 days after therapy cessation; plasma viremia subsequently declined to <1000 copies/ml for
2.6 years of follow-up. Rebound viremia was accompanied by a rapid and potent autologous nAb response by day 58, and the early virus escaped these nAbs by day 119 post therapy cessation (62).

AC13 interrupted therapy after 1 year. Plasma viremia rebounded to a peak of 3,756 copies/ml and at the subject’s request, ART was re-initiated 89 days later. Virus was previously isolated by PBMC co-culture during this second treatment phase (134 days after the first therapy interruption) (63). This virus was neutralized by plasma obtained 79 days after the first therapy interruption (55 days prior to virus isolation), suggesting minimal viral Env evolution while on therapy. In support of this, the amino acid sequence of PCR-amplified Envs at days 79 and 134 were similar (data not shown). A second supervised therapy interruption was initiated in AC13 approximately 110 days after restarting therapy. Viral load rebounded to 2,250 copies/ml and spontaneously declined to a set point of approximately 300 copies/ml without further therapy.

Both subjects were negative or weakly seropositive by ELISA (gp120, p24) and were negative for nAbs against tier 1 viruses and the autologous virus for a brief time after the first therapy interruption (63). This poor seroconversion while on therapy is consistent with potent ART-mediated viral suppression (62, 63) and is an indication that the autologous nAb responses that arose after therapy interruption is the initial response in both subjects.

Env clones from AC10 were obtained prior to ART (AC10.d0, early virus) and again on day 119 post therapy interruption (AC10.d119, escape virus). The two Envs differed at six amino acid positions in gp120 and one amino acid position in gp41 in the distal portion of the cytoplasmic tail (Fig. 2, Supplemental Figure S1). As molecularly cloned Env-pseudotyped viruses, the early AC10.d0 but not the later AC10.d119 Env was neutralized by day 58 and 93 plasma samples (Fig. 3A), confirming that the two
Envs were an autologous neutralization-sensitive and an escape variant, respectively. Autologous neutralization of the escape variant was first detected on day 128 and increased in potency to a titer of 1:465 by day 923 (Fig. 3A). The response against the AC10 early virus was either maintained or slightly declined in potency over this period of time (peak titer of 1:3,228 on day 93 declining to a titer of 1:1,251 by day 923).

Env clones from subject AC13 were obtained on day 134 (AC13.d134, early virus) and again on day 237 (AC13.d237, escape virus) after the first therapy interruption (38 days after the second therapy interruption). The two Envs differed by three amino acids in gp120 and two amino acids in gp41 (Fig. 2, Supplemental Figure S2). A pseudovirus bearing the AC13.d134 early Env was very sensitive to autologous neutralization by plasma at multiple time points whereas a pseudovirus bearing the later AC13.d237 Env was completely resistant except for a transient weak response detected on days 267 and 342 (Fig. 3B). Thus, as for AC10, the two Envs chosen for study from subject AC13 were derived from an autologous neutralization-sensitive and a later escape variant, respectively. Interestingly, plasma samples from subject AC13 also neutralized the AC10 early virus but not the AC10 escape virus (Fig. 3C), suggesting that a similar epitope was targeted in both subjects. We did not detect reciprocal neutralization of the AC13 viruses by plasma from subject AC10 (Table 1).

**Identification of amino acids in gp120 associated with neutralization escape.** Site directed mutagenesis was used to identify specific escape mutations. Sequence-verified mutant Envs were co-transfected with an Env-defective backbone plasmid in 293T cells and the resulting Env-pseudotyped viruses were tested for neutralization by autologous plasma samples. Mutating the AC10 early virus to contain the I415T mutation found in the escape variant reduced the sensitivity of the virus to autologous neutralization by only 2.2-fold (Table 1). However, in a reciprocal experiment, reversion to T415I in the
AC10 escape virus conferred a high level of sensitivity to autologous neutralization (at least a 20-fold increase, Table 1). I415T creates a sequon for potential N-linked glycosylation at position 413 in the C-terminus of V4 (Fig. 2, Supplemental Figure S1). Mutating position 413 to remove the sequon in the AC10 escape virus (N413A mutation) had the same effect as the T415 mutation, rendering the virus highly sensitive to autologous neutralization (Table 1) as further evidence that this N-linked glycan mediated escape. Separate introductions of EGK-N185D, K343E, A388T, T461N, N477D and K826T into the AC10 early virus produced little or no effect on autologous neutralization (Table 1), reinforcing the dominant effect of position 415 and illustrating that not all mutations found in the escape virus were required for escape.

Among the mutations found in the AC13 escape variant, K295E immediately N-terminal to the V3 loop of gp120 was mainly responsible for escape. Thus, introducing K295E into the AC13 early virus conferred resistance to autologous neutralization, while the reciprocal E295K mutation rendered the AC13 escape virus highly sensitive to autologous neutralization (Table 1). Two additional mutations arose in the gp120 of the AC13 escape virus: M100V in the \( \alpha_1 \) helix, and K183E in V2. Reversion of the escape virus to V100M had no effect on autologous neutralization (Table 1). Reversion to E183K had a modest effect, slightly increasing sensitivity, with a further minor increase when the V100M reversion was combined with E183K. The double reversion E183K+E295K resulted in a marginal increase in sensitivity compared to E295K alone. The V100M+E183K+E295K triple mutation increased sensitivity further, surpassing that of the early AC13 virus. These results confirm that K295E was mainly responsible for escape in AC13, with minor contributions from M100V and K183E that were partially dependent on K295E.
Because AC13 plasma cross-neutralized the AC10 early virus, we tested whether AC13 plasma would neutralize any of the site-directed AC10 mutant viruses. Positions 415 and 295 had strong effects (Table 1). Mutating the AC10 early virus to contain I415T conferred resistance, whereas reversion to T415I in the AC10 escape virus conferred a high level of sensitivity. This was similar to AC10 plasma (Table 1) and suggested that position 415 is important for neutralization by plasma from both subjects. We also tested whether the K295E mutation that was responsible for escape in AC13 would render the early AC10 virus resistant to neutralization. The early and escape viruses from AC10 both contained a K at this position. Nonetheless, introducing K295E into the AC10 early virus rendered the virus resistant to AC13 plasma and markedly less sensitivity to AC10 plasma. Overall these observations are additional evidence that the response in both subjects targeted the same region of gp120.

We tested whether the general neutralization phenotype of the viruses was affected by the mutations studied here. Parental and mutant viruses were assayed with a panel of heterologous HIV-1-positive serum samples and IgG pools (HIVIG and HIVIG-C). Only two mutant viruses exhibited a substantial difference in neutralization phenotype compared to the parental virus (Table S1). Thus the E183K and E183K+E295K mutations introduced in the AC13 escape virus conferred enhanced sensitivity. The level of enhanced sensitivity was equivalent in both cases, suggesting that E183K was solely responsible for the effect. E183K had little effect on autologous neutralization of the early AC10 and AC13 viruses and therefore was rarely included in our assessments of mutant viruses. These results indicate that key mutations used in our epitope mapping experiments did not induce a general change in the neutralization phenotype of the virus as measured with heterologous antibodies.

**Epitope mapping with autologous neutralizing mAbs.** The polyclonal nature of plasma nAbs might complicate the identification of epitope-specific responses in cases where more than one nAb
specificity is present. A possible example was the T415I mutation (potential glycan masking mechanism) that rendered the AC10 escape virus highly sensitive to autologous neutralization, whereas the reciprocal I415T mutation had only a modest effect on reducing the sensitivity of the AC10 early virus. This apparent discrepancy might be explained by the presence two or more distinct nAbs against different epitopes on the early virus, only one epitope of which is shared with the escape virus and is masked by the glycan. For a more refined analysis we used mAbs derived from cryopreserved B cells from AC10 and AC13. The mAb 5.9C from subject AC10 neutralized the AC10 early virus but not the corresponding escape virus (Fig. 3D). As with AC10 plasma, 5.9C it did not neutralize the early and escape viruses from subject AC13 (Table 1). The mAbs LG1.1C and LG3.2A from subject AC13 neutralized the early viruses from both AC10 and AC13, with greatest activity against the autologous AC13 early virus and no activity against either escape virus (Fig. 3E and F). Thus, the mAbs from these subjects largely recapitulated the activity of the corresponding plasmas, confirming that autologous neutralization and escape had occurred in both subjects, and that nAbs in subject AC13 can cross-neutralize the AC10 early virus.

Because LG1.1C and LG3.2A were recovered well after escape occurred (Fig. 1), both mAbs might be expected to neutralize the early and escape viruses from subject AC13. Only LG1.1C demonstrated an ability to do this but with 100-fold lower potency against the escape virus than against the early virus (Fig. 3E, Table 1). The neutralizing activity of all three mAbs was relatively limited to the AC10 and AC13 viruses in so much as no neutralization was detected against a multi-subtype panel of 34 heterologous HIV-1 viruses (see Materials and Methods for a list of the viruses tested).

Critical residues for the epitopes of these three mAbs were probed by using site-directed mutant viruses (Table 1). In contrast to what was observed with polyclonal plasma, the AC10 early virus
acquired a high level of resistance to 5.9C when it carried the I415T mutation that potentially adds an N-linked glycan in V4. Consistent with our results with polyclonal plasma, the reciprocal T415I mutation was sufficient to render the AC10 escape virus highly sensitive to mAb 5.9C. These results support an escape pathway in subject AC10 that involved the addition of an N-linked glycan in V4. The presence in plasma of one or more additional nAbs against another region of Env might explain why the I415T mutation did not render the AC10 early virus completely resistant to neutralization by autologous plasma (Table 1).

Because the K295E mutation that led to escape in AC13 rendered the early viruses from AC10 substantially less sensitive to autologous plasma neutralization, we tested whether 5.9C from subject AC10 would be affected by this mutation. Despite the absence of this mutation in the escape virus that arose in AC10, mutation of the AC10 early virus to contain K295E rendered the virus completely resistant to 5.9C (Table 1), further suggesting that the autologous nAb response in both subjects targeted a similar epitope. We did not assay 5.9C against the AC13 series of mutant viruses because the mAb possessed no neutralizing activity against the early and escape viruses from this subject (Table 1).

Mutations that strongly influenced the neutralizing activity of the LG3.2A and LG1.1C mAbs from AC13 were mostly confined to positions 295 and 415 (Table 1). Both mAbs lost their ability to neutralize the AC10 and AC13 early viruses when the viruses were mutated to contain the resistance-associated K295E. Moreover, the reciprocal E295K mutation rendered the AC13 escape virus highly sensitive to both mAbs. Both mAbs lost their ability to neutralize the AC10 early virus when the virus was mutated to contain the resistance-associated I415T, while the reciprocal T415I mutation rendered the AC10 escape virus highly sensitive to LG3.2A. To determine whether the T415I mutation might be acting on an N-linked glycan at position 413, we introduced an alanine at position 413 to remove the glycan in the
AC10 escape virus and showed that the mutant virus acquired a high level of sensitivity to LG1.1C and 5.9C (Table 1) (LG3.2A was not tested because our supply of the mAb had been exhausted).

Collectively the results suggest that 5.9C, LG1.1C and LG3.2A bind overlapping epitopes in the spatial vicinity of position 295 adjacent to the N-terminal cysteine of the V3 loop, with a possible masking mechanism that involves the addition of an N-linked glycan at position 413 in V4. Indeed, 5.9C and LG1.1C competed with one another for binding to immobilized AC10.d0 gp120 in an ELISA format (Fig. 4) (LG3.2A could not be tested because our supply was exhausted). The control mAbs, 1.7B and 1.4E (see Materials and Methods), only blocked themselves, confirming the specificity of competitive binding. Despite their competitive binding, 5.9C and LG1.1C often exhibited disparate neutralizing activity against the AC10 series of mutant viruses (Table 1). This suggests subtle differences in the epitopes recognized by 5.9C and LG1.1C. Both mAbs exhibited identical qualitative neutralization profiles against the AC10 series of mutant viruses (Table 1), suggesting a very similar epitope recognition in this case. The neutralizing activity of LG1.1C against the AC13 series of mutant viruses closely resembled autologous AC13 plasma, including minor effects of mutations at positions 100 and 183, especially when these mutations were combined with a mutation at position 295 (Table 1).

Sequence analysis of the VH and VL genes was performed for 5.9C and showed that the mAb utilized 2-26 VH and V\_3-9 VL and was not heavily mutated (Table 2). This gene usage indicates that 5.9C is not clonally related to the autologous nAbs described previously (54). Sequencing for LG3.2A and LG1.1C was not possible due to loss of the cell line (LG3.2A) and instability of the cell line after long-term storage (LG1.1C).
**Epitope mapping by alanine scanning.** To further map the epitopes of LG1.1C and LG3.2A, we introduced alanine substitutions at sites spanning position 286 in C2 through position 357 in C3 of the AC13 early virus. Results of neutralization assays identified four additional sites that were critical to the epitopes. Alanine substitutions at positions 332 and 334 caused substantial reductions in sensitivity to LG1.1C but had no effect on LG3.2A (Fig. 5). These two positions are adjacent to the C-terminal cysteine of V3, just outside the loop, and both affect a sequon for an N-linked glycan at position 332. Interestingly, the presence of the sequon was required for neutralization in this case. The sequon was also required for the ability of mAbs LG3.2A and 5.9C to neutralize the AC10 early virus (Table 1).

Results with LG3.2A identified two additional sites (positions 293 and 337) that had modest effects (Fig. 5). Both sites are located in close proximity to sites 295, 332, 334 on either side of the V3 loop. LG1.1C and LG3.2A were not affected by alanine substitutions surrounding the V3 loop and within the V3 loop, including the crown of the loop. Overall these results are additional evidence that mAbs LG1.1C, LG3.2A and 5.9C recognize the same region of gp120 where depending on the virus, an N-linked glycan at position 332 might be required for neutralization.

The combined results of site-directed mutagenesis and alanine scanning suggest that a region just below the base of the V3 loop and comprising flanking regions in C2 and C3 (α2-helix), was the target for autologous neutralization in subjects AC10 and AC13 (Fig. 6). In some cases the presence of an N-linked glycan at position 332 was strictly required. Although extensive alanine scanning was not performed on the AC10 viruses, the fact that LG1.1C and LG3.2A both neutralize the early but not late virus from AC10 (Fig. 3E and F) suggests that a similar region on gp120 was a neutralization target on the AC10 early virus. Indeed, early viruses from both AC10 and AC13 mutated to contain K295E resisted neutralization by LG3.2A (Table 1). Both viruses contain a sequon for N-linked glycosylation at position 332 that was required for neutralization by the mAbs (Supplemental Figures S1 and S2). Although
LG3.2A, LG1.1C and 5.9C required this sequon, removing the sequon had little or no effect on AC10 and AC13 plasma samples (Table 1), again suggesting the presence of more than one autologous nAb specificity in these samples.

**Molecular modeling.** A schematic linear representation of all amino acid positions examined in this study is shown in Figure 6. Most mutations that had substantial effects on the neutralizing activity of mAbs and autologous plasma samples cluster in a structurally constrained region just below the cysteine-cysteine bond that creates the V3 loop (sites 293, 295, 332, 334, 337), suggesting that this region is important for the formation of early autologous neutralization epitopes. An additional key residue (position 415) alters a potential site for N-linked glycosylation at position 413 in the C-terminus of V4. These two regions (V4 and the V3 base) are spatially close to one another on the x-ray crystal structure of ligated gp120 and are quite distant from the CD4 receptor binding site (Fig. 7A) but are proximal to the coreceptor binding domain (Fig. 7B). Molecular dynamics simulations indicate that positions 295 and 415 are approximately 9.5Å apart on ligated gp120 and can interact with each other, suggesting that addition of an N-linked glycan at position 413 in V4 could prevent antibody access to residue 295 adjacent to the base of the V3 loop. Another potential N-linked glycan at position 332 was required to maintain a neutralization-sensitive phenotype with LG1.1C, LG3.2A and 5.9C. This N-linked glycan is part of the epitope for the unusual glycan-dependent mAb 2G12 (Fig. 7C) (11, 86, 88) and is required for the broadly neutralizing activity of serum samples from elite neutralizers (69, 99). This N-linked glycan might also be important for autologous neutralization in subtype C infected individuals (81).

Residues associated with autologous neutralization and escape described thus far are located in the outer domain of the gp120 core. However, there were two additional resistance-associated
mutations in AC13 (M100V and K183E) that map to the inner domain of gp120 (Fig. 7D). M100V is at the proximal end of the \(\alpha\)-1 helix, whereas K183E is in the V2 loop. Site 183 is two residues downstream of the \(\alpha\)4\(\beta\)7 site binding site (3). These two sites are spatially apart from each other and from positions 295 and 415 in the ligated gp120. The inner domain undergoes substantial conformational changes upon binding to CD4. Therefore, it is difficult to spatially characterize sites 183 and 100 in the context of sites 295 and 415 in the native form.

Neutralization by heterologous plasma samples. We sought to determine whether heterologous sera from HIV-1-infected individuals might contain antibodies that target epitopes similar to those targeted by subjects AC10 and AC13. AC10 and AC13 early and escape viruses were assayed as Env-pseudotyped viruses with serum samples from 91 HIV-1-infected individuals (Table S2). Overall, the early and escape viruses from AC10 were equally sensitive to neutralization (\(p = 0.660\), paired t test), while the escape virus from subject AC13 was slightly more sensitive than the AC13 early virus (\(p = 0.0162\)). As might be expected, positive neutralization was detected more often with serum samples from chronically infected individuals than from individuals who were infected for approximately 1 year. Among the positive responses, nine exhibited a >3-fold difference in potency against the two AC10 viruses (clearly outside the normal range of variability in the assay). In five of these cases the early variant was more sensitive, whereas in four cases the escape variant was more sensitive. Only two samples showed a >3-fold difference in potency against the AC13 viruses and in both cases the escape variant was more sensitive. Thus, with few exceptions the escape viruses from both subjects were no less sensitive to neutralization by heterologous sera than the early viruses.

Serum from two subjects (subjects 707010323 and 713080175) were of particular interest because they exhibited much greater neutralizing activity against the AC10 early virus than against the
AC10 escape variant. Neutralizing Abs in these two subjects appeared to target the same region of gp120 that was targeted by AC10 in so much as the K295E and I415T variants of the AC10 early virus were far less sensitive to neutralization (Fig. 8). As controls, serum samples from three additional subjects (subjects 703010269, 703010468 and 703010547), whose serum neutralized the early and escape viruses equally well, were unaffected by the K295E and I415T mutations (data not shown), indicating that the effects seen with serum from subjects 707010323 and 713080175 were not random. Serum from subjects 707010323 and 713080175 exhibited weak or no detectable activity against a panel of heterologous subtype B and C viruses (6535.3 QH0692.42, SC422661.8, PVO.4, AC10.0.29, RHPA4259.7, Du156.12, Du172.17, Du422.1, ZM197M.PB7, ZM214M.PL15, CAP45.2.00.G3) as evidence that the response was narrow in specificity, much the same as in subjects AC10 and AC13. Subject 707010323 is a chronically infected individual in Tanzania. Subject 713080175 is a chronically infected individual in the United Kingdom.

DISCUSSION

We describe the epitopes of the initial autologous nAb response in two HIV-1 subtype B-infected individuals (AC10 and AC13). Complete gp160 genes from two time points from each individual were cloned, sequenced and characterized as Env-pseudotyped viruses in neutralization assays with autologous plasma samples and mAbs. Autologous neutralization and escape was demonstrated in both cases and was associated with very few mutations in gp120 and gp41. Detailed analysis of these mutations by site-directed mutagenesis, alanine scanning and molecular modeling identified a region just below the base of the V3 loop, near the coreceptor binding domain of gp120, as the most likely target for autologous neutralization in both individuals. Multiple overlapping epitopes in this region appeared to be targeted, often with a shared requirement for K295 in C2 flanking the N-terminus of the V3 loop, and also the presence of an N-linked glycan at position 332 in C3, immediately C-terminal to V3.
In some cases the epitopes appeared to be susceptible to shielding by an N-linked glycan at position 413 in V4. A limited survey identified nAbs with similar epitope specificity in two unrelated individuals from other countries.

Although the same region of gp120 was targeted in both subjects, the mechanism of escape was different. Escape in AC13 involved a single mutation at position 295 that is a likely contact residue. A K295E mutation led to escape from autologous plasma and from autologous mAbs LG1.1C and LG3.2A. Position 295 on subtype B viruses is frequently occupied by an N-linked glycan that participates in the epitope for 2G12 (86, 88). The absence of this glycan on the early and escape viruses from subjects AC10 and AC13 likely explains why the viruses are resistant to 2G12 (data not shown), and may also explain why this region could be targeted by the autologous nAb response in these two individuals.

Interestingly, the single K295E mutation that was responsible for escape in AC13 also could mediate autologous neutralization resistance in subject AC10, even though this mutation was not found in the AC10 escape virus. Indeed, this single site produced the most dramatic effect on autologous neutralization of viruses from both subjects. K to E changes at other sites in gp120 have been associated with resistance to autologous nAbs in subtype C infection (67, 80), suggesting a common amino acid substitution for escape. K (lysine) to E (glutamic acid) results in a positive to negative charge reversal that could alter the conformation of Env spikes and disrupt electrostatic interactions between nAbs and their cognate epitopes (28). Given the highly glycosylated nature of HIV-1 Env, it is noteworthy that charged residues preferentially interact with glycans, where mannose sugars in glycans can make hydrogen bond interactions with both acidic and basic residues (40).
Escape in AC10 was associated with a single mutation at position 415 that potentially adds an N-linked glycan at position 413 in the C-terminus of V4. Adding this glycan to the early virus by site directed mutagenesis had little effect on escape from autologous plasma; however, this single change resulted in complete escape from mAb 5.9C as evidence for potent escape from at least this one autologous nAb specificity. The presence of one or more additional, unidentified nAb specificities in this subject might explain the lack of complete escape from autologous plasma. However, in reciprocal experiments, removing this glycan from the AC10 escape virus conferred a high level of sensitivity to both 5.9C and autologous plasma (Table 1). This latter observation suggests that the 413 glycan can differentially shield multiple overlapping epitopes, where perhaps the extent of shielding depends on other mutations in the escape virus that position the glycan for optimal shielding.

One piece of evidence suggested that the 413 glycan is not part of a shielding mechanism. When the T415I mutation was used to remove the putative glycan from the AC10 escape virus, the virus remained resistant to the heterologous mAb LG1.1C; however, when the N413A mutation was used, the virus became highly sensitive to this mAb (Table 1). Both mutations should have had the same effect on neutralization if the glycan was masking the target epitope. It is possible that this site is not glycosylated even though the sequon is present, in which case one or more amino acids in this region could participate in the epitope directly, or be required for the proper conformation of the epitope. It is also possible that LG1.1C requires contact with a core residue at position 415, whereas the other mAbs do not. This latter case would be consistent with our observation that the profile of neutralization against a series of mutated viruses was quite similar with LG3.2A and 5.9C, whereas a much different profile was seen with LG1.1C (Table 1).
If the autologous nAb response in both subjects targeted the same region of gp120, why then was the mechanism of escape different? The answer might lie in the size and structure of the V4 loop. The early and escape viruses from AC13 both contain 6 N-linked glycosylation sites in V4, whereas the early AC10 virus encodes only 3 sites until the I415T mutation added a fourth in the escape variant (Supplemental Figures S1 and S2). Moreover, the early viruses from AC10 and AC13 differ by approximately 20% in their gp160 amino acid sequences, including differences in the size of V4 and the number and location of N-linked glycans in V4 (Supplemental Figures S1 and S2). Opportunities for escape might have relied on a particular V4 configuration that was present in AC10 but not AC13, although we do not exclude the possibility that another mechanism was responsible.

Similar mechanisms of escape have been described previously (54, 65-67, 80-82) but to our knowledge, the region we identified as a target for autologous nAbs has not been previously demonstrated in HIV-1 infection. Epitopes for autologous neutralization in AC10 and AC13 were not entirely identical in so much as plasma samples and mAbs from both subjects did not always cross-neutralize the other subject’s early virus. Moreover, the two MAbs from subject AC13 (LG1.1C and LG3.2A) exhibited substantial differences in their ability to neutralize several mutant viruses (Table 1), suggesting a polyclonal B cell response to overlapping epitopes in this subject. A highly focused autologous polyclonal nAb response has also been described for a HIV-1 subtype C-infected seroconverter (54).

An earlier study of autologous nAbs in subtype B-infected individuals highlighted the importance of N-linked glycans in an evolving glycan shield model of escape (100). In that study, several sites of N-linked glycosylation in V4 were confirmed by site-directed mutagenesis to be involved in escape; however, the sites they identified are distinct from site 413 identified here. Interestingly, N-linked
glycosylation at position 413 was highlighted in two recent studies that identified mutations that were strongly associated with Envs from individuals who mounted particularly potent nAb responses (29, 39). While this association was highly significant, the results could reflect either recurrent escape from potent nAbs, or an Env that was better able to elicit nAbs.

Epitope masking was not the only possible role for N-linked glycans in our study. Alanine scanning revealed that mAbs LG1.1C, LG3.2A and 5.9C require a sequon that potentially adds an N-linked glycan at position 332. As ‘self’ structures, glycan moieties would be unusual targets of a mature B cell response. Nonetheless, this same sequon was required for the broadly neutralizing activity of serum samples from 5 of 19 elite neutralizers (99), where the broadly neutralizing activity of serum from one of these elite neutralizers was sensitive to mutations at positions 295 and 332 and could be absorbed with a heavily N-glycosylated yeast protein (TM-Pst1), suggesting that the epitope was comprised at least in part of an oligomannose cluster. In other studies, a potential N-linked glycosylation at position 332 was required for the broadly neutralizing activity of serum samples from 2 of 17 subjects (69) and from 3 of 9 subjects (Tomaras et al., manuscript submitted). Thus, altogether the sequon at position 332 might be a relatively common component for autologous and heterologous neutralization in a subset of individuals.

A possible shared requirement for the N-linked glycan at position 332 for early autologous nAbs and for broadly nAbs suggests that in some cases both types of responses can focus on the same region of gp120. Additional proof will come if a broadly neutralizing, 332 glycan-dependent mAb is discovered. Alternatively, this particular broadly neutralizing serum activity might involve multiple overlapping Ab specificities that share a common glycan requirement but are not broadly neutralizing individually. Other N-linked glycans on gp120 also appear important for nAbs. In addition to contacting core
residues, mAb VRC01 makes contact with a glycan at position 276 on gp120 (105). N-linked glycans in conserved regions of V2 and V3 are required for the broadly neutralizing activity of mAbs PG9 and PG16 (21, 98). A potential glycan at position 339 has also been shown to be important for autologous neutralization in a subtype C-infected subject, CAP88 (67). In the only other studies of autologous neutralizing mAbs, neutralization required the presence of an N-linked glycan in V1 (54, 82). In these latter cases, as in our study, it is uncertain whether the epitopes are comprised in part of carbohydrate, or whether the N-linked glycans are mostly affecting protein conformation (21, 45, 48).

Mapping of sites 295, 332 and 415 on three-dimensional structures and profiling the contacts from molecular dynamics simulations, point to a region encompassing the β12, β13 and β19 strands of gp120 as the target for autologous neutralization in subjects AC10 and AC13. As shown in figure 9A, these three sites occur in very close proximity (in a row) in the stable four stranded anti-parallel sheet of the outer domain of ligated HIV-1 gp120. This spatial proximity is maintained both in the ligated and unligated forms of gp120 (Fig. 9A and B). Even though the beta sheet topology is not maintained to the same level as in the ligated form, figure 9B shows that the sites map to a spatially proximal region in the unligated SIV gp120. We used the unligated SIVgp120 structure here because this structure is based on a glycosylated protein (15); all other available X-ray structures of gp120 are deglycosylated (20, 37, 42, 43, 70, 106). The glycosylated SIVgp120 3-D structure allowed us to address a potential role of glycans in the area of interest, where one glycan would be required to maintain the epitope and another glycan would confer escape. In that X-ray structure, the only site that is glycosylated in the region of interest is residue 446 (HXB2 numbering). Figure 9C and D show how a glycan at site 446 in the β22 strand could affect neighboring sites in the β12, β13 and β19 strands. This experimentally determined glycan orientation suggests two possible scenarios. First, the base of a glycan could directly interact with a residue from a neighboring strand, where the tip of the glycan tends to extend outwards. Second,
glycans are relatively large and can cover neighboring beta strands and potentially interact with glycans originating from these strands. In a similar way, the base of a glycan at position 332 might directly interact with the charged residue at site 295 from the neighboring strand. In this case, a mutation at site 295 could change the projection of the glycan at site 332 that is necessary to maintain the epitope. Introduction of a glycan at site 413 could lead to glycan-glycan interactions between sites 413 and 332 and thereby modify the conformation of the epitope. Here, the interactions are expected to occur at the tip of the glycans. The 2G12 epitope is an example where tips of glycans from distant sites come together to interact. Alternatively, it is possible that the glycan from 413 covers neighboring beta-strand regions as shown in figure 9D and blocks antibody access.

Most previous studies of the epitopes of the initial autologous nAb response in HIV-1 infection focused on subtype C viruses. The most frequent epitopes recognized in subtype C infection are located in the V1V2 loop (54, 65, 67, 80, 82), with additional epitopes in C3 (α2 helix), V4, V5 and other unidentified regions (65, 67, 81, 82). In our study, a mutation at position 183 in the V2 loop had a small but detectable influence on the neutralizing activity of autologous plasma and mAb LG1.1C (Table 1), whereas it had a more profound effect on the neutralizing activity of heterologous serum samples (Supplemental Table S1). This site is spatially distant from the base of the V3-loop (Fig. 7D), suggesting a mild allosteric effect on autologous neutralization. A mutation in V5 was present in the AC10 escape variant but this mutation had no effect on autologous neutralization. Neither escape virus contained mutations in C3; however, alanine scanning identified position 337 in the α2 helix as having a modest effect on the neutralizing activity of LG3.2A (Fig. 5).

Our interpretations regarding the epitope specificity and mechanism of escape from autologous nAbs in subjects AC10 and AC13 are inferred from a detailed analysis of viral Envs by site-directed
mutagenesis, alanine scanning and molecular modeling. Although our interpretations are plausible, we acknowledge certain limitations when studying epitopes that may be conformation-dependent and subject to allosteric effects arising from distant sites. Indeed, cyclized and non-cyclized versions of a peptide covering the entire V3 loop and containing relevant regions on both sides of the loop, failed to compete for autologous neutralization and were not bound by LG3.2A (data not shown), suggesting that the epitopes we describe are non-linear. We also acknowledge that our interpretations regarding N-linked glycans at positions 332 and 413 are based on the presence or absence of the sequon rather than direct biochemical evidence that the sites are indeed glycosylated. Nonetheless, our evidence for one or more novel autologous nAb epitopes below the base of the V3 loop is consistent with mapping studies of nAbs in HIV-1-positive sera that have been unable to identify the determinants of all neutralization activity (5, 49, 69, 89). Our observations, together with autologous nAb epitopes identified by others and as yet undiscovered epitopes, might be important constituents of NAbs that will coalesce into an effective vaccine strategy.

ACKNOWLEDGEMENTS

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Table 1. Neutralization analysis of parental and mutant viruses.

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<th>Virus</th>
<th>ID50 (dilution)</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AC10 plasma&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AC13 plasma&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AC10.d0</td>
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<td>84</td>
</tr>
<tr>
<td>AC10.d119</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>AC13.d134</td>
<td>&lt;20</td>
<td>705</td>
</tr>
<tr>
<td>AC13.d237</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>AC10.d0 I415T</td>
<td>968</td>
<td>&lt;20</td>
</tr>
<tr>
<td>AC10.d119 T415I</td>
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<td>545</td>
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<tr>
<td>AC10.d119 N413A</td>
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<td>338</td>
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<tr>
<td>AC10.d0 ∆EGK-N185D</td>
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<tr>
<td>AC13.d237 V100M + E183K + E295K</td>
<td>nt</td>
<td>1,180</td>
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</tbody>
</table>

<sup>a</sup>Parental and mutant viruses were assayed as Env-pseudotyped viruses in TZM-bl cells. All viruses except three were created as site-directed mutants that carried the residue of the corresponding early or escape virus. Three viruses were products of alanine scanning (AC10.d119 N413A, AC10.0 N332A and AC10.d0 S334A).

<sup>b</sup>AC10 plasma was obtained 93 days post therapy interruption.

<sup>c</sup>AC13 plasma was obtained 38 days post second therapy interruption (237 days post first therapy interruption). nt, not tested.

<sup>d</sup>nt, not tested.
Table 2. Variable-heavy and variable-light chain genes of mAb 5.9C.

<table>
<thead>
<tr>
<th>mAb</th>
<th>V</th>
<th>D</th>
<th>J</th>
<th>Mutation frequency</th>
<th>CDR3 length</th>
<th>Isotype</th>
<th>V</th>
<th>J</th>
<th>Mutation frequency</th>
<th>CDR3 length</th>
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</thead>
<tbody>
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<td>4-17</td>
<td>6</td>
<td>4.0%</td>
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<td>IgG1</td>
<td>V</td>
<td>3-9</td>
<td>5.4%</td>
<td>9</td>
</tr>
</tbody>
</table>

*Genes for the variable (V), diversity (D) and joining (J) regions are shown. CDR3 lengths are the number of amino acids.*
FIGURE LEGENDS

FIG 1. Time points for plasma collection, virus isolation and mAb recovery in subjects AC10 and AC13. Arrows below the horizontal line are plasma collection time points. Above the horizontal lines, labels for 5.9C, LG1.1C and LG3.2A indicate the sampling time point at which these mAbs were isolated.

FIG. 2. Amino acid differences between the autologous neutralization-sensitive and escape variants from subjects AC10 and AC13. The gp120 and gp41 subregions, the variable regions of gp120 and the membrane spanning domain (msd) of gp41 are indicated above the schematics. Mutations and their location in the escape variants are shown with arrows below the schematics. PNLG, potential N-linked glycan.

FIG 3. Autologous neutralization and escape in AC10 and AC13. The early and escape viruses from subjects AC10 and AC13 were assayed with autologous plasma samples and mAbs 5.9C, LG1.1C and LG3.2A. Plasma samples were obtained at the indicated days post first therapy interruption. ID50 values for plasma samples are the reciprocal dilution that resulted in 50% neutralization. ID50 values for mAbs are expressed as µg/ml, where lower values indicate stronger neutralizing activity.

FIG. 4. Cross-competition analysis of LG1.1C and 5.9C binding to gp120. The ability of unlabeled mAb to compete the binding of biotin-labeled mAb to AC10.0.29gp120 was determined by ELISA. Percent binding of the biotin-labeled mAb was calculated relative to binding in the absence of the unlabeled competitor. Unlabeled mAbs are shown as white dotted bars (1.7B), black bars (LG1.1C), gray bars (5.9C) and white hatched bars (1.4E).
FIG. 5. Effect of alanine substitutions. Alanine substitutions were introduced at multiple sites in the gp120 of AC13.d134. Env-pseudotyped viruses containing a single alanine mutation were examined for their susceptibility to neutralization by LG1.1C (Top) and LG3.2A (bottom). Substitutions that caused a substantial decrease in sensitivity are denoted at the top of the corresponding bar.

FIG. 6. Graphical representation of the combined results of site directed mutagenesis and alanine scanning experiments. The V3 loop and surrounding residues are shown for the AC13 early virus, whereas the V4 loop is shown for the AC10 early virus. Sites that were critical for neutralization by a particular mAb are color-coded as indicated. A likely antibody contact region comprising the β12 and β13 sheets is shaded (see also Fig. 9).

FIG. 7. Location of critical mutations on the ligated X-ray crystal structure of gp120 (PDB code: 1RZK). The gp120 molecule is marked in blue and the CD4 and 17B binding sites are marked in green. The contact regions in gp120 are marked in white. A. Spatial location of sites 295 and 415 (red balls) with respect to the CD4 binding site. B. Spatial location of sites 295 and 415 (red balls) with respect to the CCR5 and 17b binding site. Silver balls in B correspond to key sites that have been shown through mutations to be critical for coreceptor binding. C. Sites 295 and 415 (red balls) in the context of 2G12 epitope sites (green balls). All sites including 295 and 415 are exposed on the surface of the molecule. D. Two additional resistance-associated sites (100 and 183) in AC13 (marked as green balls) that occur in the inner domain of gp120. Site 100 is shown for clarity in a modeled V1/V2 loop for visual purposes only.

FIG. 8. Escape mutations in subjects AC10 and AC13 affect the neutralizing activity of heterologous HIV-1-positive serum samples. Serum samples from two chronically HIV-1-infected individuals were assayed.
for neutralizing activity against the early and late viruses from subject AC10 and against mutants of the early virus that contained escape mutations at positions 295 and 415.

Fig. 9. Region of interest in the outer domain of gp120 as a possible target for autologous neutralization. Region (yellow) and key sites (red and green balls) are marked in ligated (A) and unligated (B) gp120. Ligated gp120 structure corresponds to JRFL strain with the V3 loop but lacking V1/V2 loops (PDB code: 2B4C). The unligated gp120 structure corresponds to SIV gp120 core lacking V1/V2 and V3 loops (PDB code: 2BF1). We also highlight site 446 in (B) because it is the only site with a glycan in that region. (C) and (D) show the projection of a glycan at site 446 in the β22 strand of unligated gp120 in two orientations for visual clarity.
Fig. 1
Tang et al.
Fig. 3
Tang et al.
Fig. 4
Tang et al.
Fig. 6
Tang et al.
Fig. 7
Tang et al.
Fig. 8
Tang et al.