Balancing Reversion of Cytotoxic T-Lymphocyte and Neutralizing Antibody Escape Mutations within Human Immunodeficiency Virus Type 1 Env upon Transmission

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Human immunodeficiency virus type 1 (HIV-1) envelope protein (Env) is subject to both neutralizing antibody (NAb) and CD8 T-cell (cytotoxic T-lymphocyte [CTL]) immune pressure. We studied the reversion of the Env CTL escape mutant virus to the wild type and the relationship between the reversion of CTL mutations with N-linked glycosylation site (NLGS)-driven NAb escape in pigtailed macaques. Env CTL mutations either did not revert to the wild type or only transiently reverted 5 to 7 weeks after infection. The CTL escape mutant reversion was coincident, for the same viral clones, with the loss of NLGS mutations. At one site studied, both CTL and NLGS mutations were needed to confer NAb escape. We conclude that CTL and NAb escape within Env can be tightly linked, suggesting opportunities to induce effective multicomponent anti-Env immunity.

CD8 T-cell responses against human immunodeficiency virus (HIV) have long been observed to select for viral variants that avoid cytotoxic T-lymphocyte (CTL) recognition (2, 5, 15, 18, 27). These immune escape mutations may, however, result in reduced replication competence (“fitness cost”) (11, 20, 26). CTL escape variants have been shown to revert to the wild type (WT) upon passage to major histocompatibility complex-mismatched hosts, both in macaques with simian immunodeficiency virus (SIV) or chimeric SIV/HIV (SHIV) infection (11, 12) and in humans with HIV type 1 (HIV-1) infection (1, 19).

Most analyses of CTL escape and reversion have studied Gag CTL epitopes known to facilitate control of viremia (7, 14, 21, 30). Fewer analyses have studied Env-specific CTL epitopes. Recent sequencing studies suggest the potential for mutations within predicted HIV-1 Env-specific CTL epitopes to undergo reversion to the WT (16, 23). Env-specific CTL responses may, however, have less impact on viral control of both HIV-1 and SIV/SHIV than do Gag CTL responses (17, 24, 25), presumably reflecting either less-potent inhibition of viral replication or minimal fitness cost of escape (9).

Serial viral escape from antibody pressure also occurs in both macaques and humans (3, 13, 28). Extensively glycosylated, and this “evolving glycan shield” can sterically block antibody binding without mutation at the antibody-binding site (8, 16, 31). Mutations at glycosylation sites, as well as other mutations, are associated with escape from neutralizing antibody (NAb) responses (4, 13, 29). Mutations in the amino acid sequences of N-linked glycosylation sites (NLGS) can alter the packing of the glycan cloud that surrounds the virion, by a loss, gain, or shift of an NLGS (32), thus facilitating NAb escape.

Env is the only viral protein targeted by both CTL and NAb responses. The serial viral escape from both Env-specific CTL and NAb responses could have implications for viral fitness and the reversion of multiple mutations upon transmission to naïve hosts.

We previously identified three common HIV-1 Env-specific CD8 T cell epitopes, RY8<sub>788-795</sub>, SP9<sub>110-118</sub>, and NL9<sub>671-679</sub> and their immune escape patterns in pigtail macaques (Macaca nemestrina) infected with SHIV<sub>mac239</sub> (25). SHIV<sub>mac239</sub> is a chimeric virus constructed from an SIV<sub>mac239</sub> backbone and an HIV-1<sub>HXB2</sub> env fragment that was passaged through macaques to become pathogenic (11). This earlier work provided an opportunity for detailed studies of how viruses with Env-specific CTL escape mutations, as well as mutations in adjacent NLGS, evolve when transmitted to naïve pigtail macaques.

Reversion of Env CTL escape mutant viruses. Two passaged CXCR4-tropic SHIV<sub>mn229</sub> viruses, which had mutated at three previously described Env CTL epitopes, RY8, SP9, and NL9 (25), were inoculated intravenously into four naïve pigtail macaques using 5 × 10<sup>6</sup> peripheral blood mononuclear cells and 1 ml of plasma from donor animals as previously described (22). This small amount of plasma would not be expected to transfer durable neutralizing capacity to recipient macaques. Macaque 6279 (at week 11 after SHIV<sub>mn229</sub> infection) was the donor animal for RY8 reversion studies of recipient animals 6255 and 6238. Macaque 5350 (at week 29 after SHIV<sub>mn229</sub> infection) was the donor animal for SP9 and NL9 reversion studies of recipient animals 5613 and 0608. All four macaques previously identified as having CTL responses to SP9/NL9 shared the Mane-B<sup>02</sup> class I allele (data not shown), so recipient animals chosen for reversion at the SP9/NL9 epitopes (0608 and 5613) were Mane-B<sup>02</sup> negative. We first studied reversion of virus mutated at the RY8 CTL epitope (located in the cytoplasmic domain of gp41). We then analyzed reversion...
...of virus mutated at both the SP9 (located in conserved region 1 of gp120) and NL9 (located in the ectodomain of gp41) CTL epitopes. No CTL responses to the Env CTL epitopes above background levels were detected for any of the four recipient animals at 4 to 7 weeks postinfection (<0.11% of CD8 T cells expressed gamma interferon by intracellular cytokine staining; not shown).

The virus transfer experiments were successful, with all four recipient animals demonstrating high peak plasma SHIV RNA levels and early depletion of peripheral CD4 T cells, characteristic of SHIV\textsubscript{mn229} infection (Fig. 1A). The mean viral load for all recipient animals in the post-acute phase of infection was 5.60 log\textsubscript{10} copies/ml, and the average peripheral CD4 T-cell level was 6.79% of all lymphocytes, compared to 48.5% preinfection. It is worthwhile to note that the donor animal for the RY8 reversion study, 6279, also responded to the SIV Gag epitope KP9\textsubscript{164-172}. This epitope was mutated in SHIV\textsubscript{mn229} to the less-fit K165R variant that we have previously described (10, 11). This less-fit K165R mutation in the viral population of donor animal 6279 reverted to the WT in the recipient animals, 6255 and 6238, shortly after acute infection. This potentially explains the reduced peak viral loads (but much more modest differences in set point viral load) in recipients 6255 and 6238 (receiving the K165R mutated Gag) compared to 0608 and 5613 (receiving the WT virus at the KP9 Gag epitope). All animals seroconverted to both SIV Gag/Pol antigens (not shown) and HIV-1 gp41 and gp120 Env antigens, as shown by Western blotting.

Cloning and sequencing across the RY8 gp41 Env CTL epitope revealed that the transferred plasma virus from donor 6279 contained 32 of 74 clones (43%) with a W790R mutation at the third amino acid, and 29 of 74 clones (40%) had a A792T mutation at the fifth amino acid (25). Viral sequencing of serial plasma samples from both recipients of this stock, animals 6255 and 6238, over 11 weeks of infection, showed that none of the 251 clones isolated from either animal reverted to the WT sequence at RY8. Rather, a change in balance between the two dominant mutations, A792T and W790R, occurred in the recipient animals. In both recipient animals, this change in viral quasispecies occurred following the development of Env-specific antibodies (Fig. 2). In recipient animal 6255, the A792T mutation at the fifth amino acid became the overwhelmingly dominant mutation during acute infection, with a >20% decline and then a reemergence between weeks 4 and 7 postinfection (Fig. 2, shaded in gray; Table 1). In animal 6238, the viral population carrying a A792T mutation at the fifth amino acid began as the dominant quasispecies until week 4 postinfection. This dominant species at week 4 became the subdominant species at week 5 postinfection. These changes in the cytoplasmic domain RY8 CTL epitope in the absence of an RY8 CTL response, but temporally associated with gp41 binding antibody, were curious. We speculate that changes outside...
this epitope (e.g., CTL or NAb escape or compensatory mutations elsewhere in Env) rendered the A792T and W790R mutations more or less fit in the contemporary immune environment.

To examine whether lack of reversion was common in other Env-specific CTL epitopes, we analyzed reversion to the WT sequence at the Env-specific CD8 T-cell epitope, SP9. Donor animal 5350 had 83% viral sequence of the S110G SP9 escape variant, and CD8 T cells from animal 5350 did not respond to the mutant peptide (data not shown). The virus with the S110G mutation remained the dominant viral species in both recipients through the first 4 weeks of infection (Fig. 2 and Table 1). Remarkably, viral clones from both recipient animals demonstrated a 100% switch to the WT sequence at week 5 (18 of 18 clones in both animals). Further, the S110G escape mutation reappeared 14 days later in 100% of clones (21 of 21) in both animals. This appearance and disappearance of the WT sequence occurred in both recipient animals, 5613 and 0608, and at the same time point. This striking pattern of transient SP9 reversion was temporally associated with the development of Env-specific antibodies in both animals (Fig. 2).

We then analyzed sequence evolution at the NL9 Env-specific CD8 T-cell epitope, since the donor animal, 5350, had also responded to this CTL epitope. A minor proportion (5%) of the inoculum contained the N677H NL9 mutant. However, clones isolated 1 week after infection, those with the N677H mutation together with another mutation, D674N, were the dominant clones isolated for the first 5 weeks of infection (Fig. 2 and Table 1). At week 7 postinfection, the WT sequence at NL9 reemerged in both animals. The WT sequence slowly became the dominant quasispecies in animal 5613 by week 14. However, in animal 0608, the appearance of the WT NL9 CTL sequence was transient (Fig. 2, highlighted in gray), and the EM sequence again was the sole quasispecies detected at weeks 14 and 23. Again, these changes in viral population proportions followed the development of Env-specific antibodies, as shown by Western blotting.

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**FIG. 2.** Lack of reversion of Env CTL escape mutations upon transmission. (A) At the RY8 epitope, a mix of two mutant viruses (W790R and A792T mutations) was administered to two animals (6255 and 6238). There was no reversion to the WT sequence at the CTL epitope RY8 in either recipient animal in the first reversion trial (6255 and 6238). (B) Transient reversion to the WT sequence (SLWDQSLKP) at the CTL epitope SP9 at week 5 in both recipient animals (5613 and 0608). (C) Partial reversion to the WT sequence at the CTL epitope NL9 at week 7 in both recipient animals (5613 and 0608). In all recipient animals, changes in viral quasispecies dominance at CTL epitopes occur shortly after the development of Env antibodies as measured by Western blot densitometry (gray triangles). Week 0 in all graphs represents the cloning and sequencing data from the donor plasma used. Gray shading reflects major shifts in the dominance of CTL escape mutant viruses shortly after evolution of Env antibodies.
Clonal links between NLGS changes and CTL mutations. Reversion to WT sequence at Env-specific CTL epitopes did not occur at some epitopes and was transient in others. The temporal association at 5 to 7 weeks after infection of quasi-species dominance at Env CTL epitopes and the development of antibody responses was curious, suggesting potential links between pressure applied by antibodies and the propensity for reversion at CTL epitopes. Since NAb pressure is often reflected by changes in glycosylation patterns, we analyzed sequence changes at CTL epitopes. Since NAb pressure is often reflected by changes in glycosylation patterns, we analyzed sequence changes at CTL epitopes. Since NAb pressure is often reflected by changes in glycosylation patterns, we analyzed sequence changes at CTL epitopes. Since NAb pressure is often reflected by changes in glycosylation patterns, we analyzed sequence changes at CTL epitopes. Since NAb pressure is often reflected by changes in glycosylation patterns, we analyzed sequence changes at CTL epitopes.

A common pattern emerged with the mutational motifs that arise in the NLGS 18 amino acids downstream of the SP9 CTL epitope (Table 1). In 91% of 97 clones sequenced at multiple time points from three animals (donor animal 5350 and recipient animals 0608 and 5613, not including week 0), when the SP9 CTL epitope is mutated (S110G) at the first amino acid, then the virus also has N133E and S138N mutations at both NLGS immediately downstream. At week 5 postinfection, in recipient animals 0608 and 5613, this dominating viral quasi-species disappears, precisely coincident with the transient CTL reversion. It is replaced in 100% of 18 clones by the original SHIVms229 WT sequence for the entire area of interest except for an N133D or N133E mutation in the first downstream NLGS. Two weeks later, again in both recipient animals, the mutant SP9 epitope and both mutant NLGS sequences are completely restored (Table 1).

A distinctive pattern also emerged in the same three animals

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* ID, identification.

**TABLE 1. Evolution of mutations within Env CTL epitopes and adjacent NLGS**
were constructed. Four contained combinations of the WT, the serial plasma samples. With CTL epitope mutations, affect neutralization sensitivity of NLGS, which appear to be both spatially and temporally linked (the supplemental material). We asked whether changes in the EGFP-reporter pseudovirus assay (for details, see Fig. S1 in the supplemental material). Neutralization of various WT and mutated autologous virus NLGS and CTL sites. To analyze this further, we studied NAb pressure may influence viral sequences linked across incidents sequence changes in proximal NLGS, suggested that dominance at the SP9 and NL9 CTL epitopes, and the coinfection of the double mutant pseudovirus around NL9, but not SP9, from neutralization by NAbs circulating at week 11 and 15 postinfection.

This loss of the intraepitope NLGS was always accompanied by the seemingly common T626M mutation in the upstream NLGS. We then noted that the D674N mutation in the NL9 CTL epitope mutated to N677H or reverted transiently to the WT, the intraepitope NLGS is lost (Table 1). At later time points in macaques 5350, 0608, and 5613, when the NL9 CTL epitope mutated to N677H or reverted transiently to the WT, the intraepitope NLGS is lost (Table 1). This loss of the intraepitope NLGS was always accompanied by the seemingly common T626M mutation in the upstream NLGS. We then noted that the D674N mutation in the NL9 CTL epitope was always associated with both the H625N and T626M mutations within the CTL epitope. Thus, there was a delay in neutralization of the mutated Env sequence, but only when both the CTL and NLGS mutations were present. It is notable that sequence from animal 5350 with regard to a mutation at the NLGS located 43 amino acids upstream from the NL9 CTL epitope. As the D674N mutation arose in the NL9 CTL epitope (creating a new potential NLGS within the CTL epitope), the H625N and T626M mutations also arose in the upstream NLGS. This occurred in 100% of 32 clones sequenced in the three animals (Table 1).

At later time points in macaques 5350, 0608, and 5613, when the NL9 CTL epitope mutated to N677H or reverted transiently to the WT, the intraepitope NLGS is lost (Table 1). This loss of the intraepitope NLGS was always accompanied by the seemingly common T626M mutation in the upstream NLGS. We then noted that the D674N mutation in the NL9 CTL epitope was always associated with both the H625N and T626M changes within the adjacent NLGS. Although we did not sequence clones spanning both the SP9 and NL9 sites in these animals, almost all time points had clones that were either fully the WT or that had escape mutations at either the SP9 or NL9 epitope (Fig. 2, Table 1), suggesting there were no clear links between mutations at SP9 and NL9 epitopes.

Neutralization of autologous Env sequences with CTL and NLGS changes. The timing of the swaps in viral quasispecies dominance at the SP9 and NL9 CTL epitopes, and the coincident sequence changes in proximal NLGS, suggested that NAb pressure may influence viral sequences linked across NLGS and CTL sites. To analyze this further, we studied neutralization of various WT and mutated autologous virus sequences over time in an enhanced green fluorescent protein (EGFP)-reporter pseudovirus assay (for details, see Fig. S1 in the supplemental material). We asked whether changes in the NLGS, which appear to be both spatially and temporally linked with CTL epitope mutations, affect neutralization sensitivity of serial plasma samples.

To gain insight into this question, eight Env pseudoviruses were constructed. Four contained combinations of the WT, the NL9 CTL mutation NWNITNWNL, and/or the adjacent NLGS mutation, NNMT. The other four pseudoviruses contained combinations of the WT, the SP9 CTL mutation GLWDQSLKP, and/or the two adjacent NLGS mutations, NETN-NNSS, or GLWDQSLKPNDTN-NNSS, and their adjacent NLGS. The pseudoviruses were tested for sensitivity to neutralization by serum samples collected from donor animal 5350 at the indicated postinfection time points (for a description, see Fig. S1 in the supplemental material). The warmer the color, the more potent the neutralization of the pseudovirus. Neutralization of combinations of CTL and NLGS mutations around the NL9 CTL epitope (A) and similar neutralization studies of mutations of combinations of CTL and NLGS around the SP9 CTL epitope (B) are shown. There was partial protection of the double mutant pseudovirus around NL9, but not SP9, from neutralization by NAbs circulating at week 11 and 15 postinfection.

FIG. 3. Mutations in and around NL9 CTL epitope provide partial protection from neutralization. Eight pseudoviruses were constructed that were either the WT or had a mutation at the NL9 epitope (HHTT-NWFNDITNLWNL, NNMT-NWFNTNLWNL, NNMT-NWFNTNLWNL, or HWT-NWFNTNLWNL) and SP9 epitope (SLWDQSLKP-NNDTN-NNSS, GLWDQSLKP-NETN-NNSS, SLWDQSLKP-NETN-NNSS, or GLWDQSLKP-NDTN-NNSSS) and their adjacent NLGS. The pseudoviruses were tested for sensitivity to neutralization by serum samples collected from donor animal 5350 at the indicated postinfection time points (for a description, see Fig. S1 in the supplemental material). The warmer the color, the more potent the neutralization of the pseudovirus. Neutralization of combinations of CTL and NLGS around the SP9 CTL epitope (B) are shown. There was partial protection of the double mutant pseudovirus around NL9, but not SP9, from neutralization by NAbs circulating at week 11 and 15 postinfection.
eventually changed back to \textit{wtNLG} as the infection progressed toward simian AIDS (Table 1).

A similar neutralization assay, with plasma from the same source, was undertaken, focusing on SP9 and adjacent NLGS. Regardless of the combinations of WT or mutant SP9 and NLGS expressed by these Env pseudoviruses, no protection from neutralization was evident. Unlike the case with \textit{mutNLG}, no delay of neutralization was afforded the mutated SP9 pseudoviruses (Fig. 3B). This result suggests that shifts in NLGS patterns around the SP9 epitope may be compensating for neutralization escape mutations elsewhere in Env, rather than directly leading to escape themselves.

We conclude that reversion at HIV Env-specific CTL epitopes in macaques either does not occur, is slow to occur, or occurs only transiently 5 weeks after infection. In general, this result casts doubt as to the fitness costs incurred by CTL escape mutations within Env and is consistent with a growing body of literature on the lack of utility of Env CTL responses (9, 17, 24, 25). However, we observed remarkable clonal links between sequence changes in CTL epitopes and adjacent NLGS mutations: changes within CTL epitopes coexisted on the same clones as changes in NLGS. The NLGS changes around the NL9 HIV-1 Env epitope were associated with delayed neutralization in vitro. Although admittedly limited to specific CTL and NLGS changes in SHIV\textit{mac239}-infected pigtailed macaques, this work provides insights into the multiple forces shaping the complex evolution of Env in the weeks following transmission.

Why would mutations within CTL epitopes be linked to changes within NLGS in HIV-1 Env? Several scenarios are possible. When two or more mutations (e.g., CTL and NAb escape mutations) are linked on the same viral clone and the WT virus contains none of the mutations, they may evolve together. If one mutation partially compensates for the fitness cost of another mutation, there may be a fitness barrier to revert just one mutation (6). The partial “reversion” at both CTL and NLGS mutations around the NL9 epitope, observed with recipient animals 0608 and 5613, may reflect an outgrowth of preexisting pure WT virus (at both CTL and NLGS sequences) that is then lost as NAb responses evolve during the first few weeks of infection. It is notable that the mutation within the NL9 CTL epitope, D674N, creates a potential NLGS. This CTL epitope mutation, in conjunction with the mutation at the adjacent NLGS, was required for partial antibody escape. No NLGS was present within the SP9 epitope, and changes within and around the SP9 CTL epitope did not confer direct NAb escape. Upon transmission to naive hosts, the CTL mutation may be retained in recipient animals, since (i) it is linked on the same viral clones with the NLGS changes that afford neutralization escape, (ii) Env CTL mutations may have modest fitness costs (24, 25), and (iii) some Env CTL mutations may contribute to a shifting glycan shield that becomes “fixed” by changes at adjacent glycosylation sites to resist neutralization. Although fitness costs of Env-specific CTL escape may not be substantial, we speculate that there may be opportunities to target linked combinations of cellular and humoral immunity to Env that are more difficult to escape without substantial fitness costs.

REFERENCES


