Human Immunodeficiency Virus Type 1 V1-V2 Envelope Loop Sequences Expand and Add Glycosylation Sites over the Course of Infection, and These Modifications Affect Antibody Neutralization Sensitivity†

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Over the course of infection, human immunodeficiency virus type 1 (HIV-1) continuously adapts to evade the evolving host neutralizing antibody responses. Changes in the envelope variable loop sequences, particularly the extent of glycosylation, have been implicated in antibody escape. To document modifications that potentially influence antibody susceptibility, we compared envelope variable loops 1 and 2 (V1-V2) from multiple sequences isolated at the primary phase of infection to those isolated around 2 to 3 years into the chronic phase of infection in nine women with HIV-1 subtype A. HIV-1 sequences isolated during chronic infection had significantly longer V1-V2 loops, with a significantly higher number of potential N-linked glycosylation sites, than the sequences isolated early in infection. To assess the effects of these V1-V2 changes on antibody neutralization and infectivity, we created chimeric envelope sequences, which incorporated a subject's V1-V2 sequences into a common subtype A envelope backbone and then used them to generate pseudotyped viruses. Compared to the parent virus, the introduction of a subject's early-infection V1-V2 envelope variable loops rendered the chimeric envelope more sensitive to that subject's plasma samples but only to plasma samples collected >6 months after the sequences were isolated. Neutralization was not detected with the same plasma when the early-infection V1-V2 sequences were replaced with chronic-infection V1-V2 sequences, suggesting that changes in V1-V2 contribute to antibody escape. Pseudotyped viruses with V1-V2 segments from different times in infection, however, showed no significant difference in neutralization sensitivity to heterologous pooled plasma, suggesting that viruses with V1-V2 loops from early in infection were not inherently more neutralization sensitive. Pseudotyped viruses bearing chimeric envelopes with early-infection V1-V2 sequences showed a trend in infecting cells with low CD4 concentrations more efficiently, while engineered viruses with V1-V2 sequences isolated during chronic infection were moderately better at infecting cells with low CCR5 concentrations. These studies suggest that changes within the V1-V2 envelope domains over the course of an infection influence sensitivity to autologous neutralizing antibodies and may also impact host receptor/coreceptor interactions.

Human immunodeficiency virus type 1 (HIV-1) evolves over the course of infection to escape the host immune responses. Host neutralizing antibodies target specific epitopes on the circulating viral envelope glycoproteins, but viruses evolve to circumvent these responses, leading to a succession of new antibodies and subsequent escape (20, 31, 43). With the simian immunodeficiency virus (SIV)/macaque model, studies have shown that viruses become more neutralization resistant by increasing the level and/or changing the pattern of glycosylation on their envelope glycoprotein (4, 32). Subsequent studies with the simian/human immunodeficiency virus (SHIV) and HIV-1 also indicated that viruses increase the number and/or vary the position of the carbohydrates on their envelope glycoprotein to shield themselves against the host antibody response and thus persist during the course of infection (5, 43). These studies, however, have been limited to a small number

of subjects and focused on subtype B HIV-1. It remains unclear whether this is a general evolutionary feature by which a majority of HIV-1 subjects escape the host humoral immune response.

With both SIV and HIV-1, envelope variable loops exert a major influence on antibody neutralization sensitivity. Deletions or mutations, especially those that affect glycosylated residues, within envelope variable loops 1 and 2 (V1-V2) have a profound impact on susceptibility to monoclonal antibodies and antibodies circulating in plasma (3, 5, 10, 16, 30, 32, 38). These studies suggest that changes to the V1-V2 domains may change the structure of an antibody epitope and/or the exposure of neutralization-sensitive domains important for envelope function. The V1-V2 variable loops are thought to shield the bridging sheet between the inner and outer domains of the viral envelope glycoprotein (13). The bridging sheet participates in the sequential binding of the host receptor, CD4, and a coreceptor, such as CCR5; these sequential interactions are necessary for cell entry (23). Studies have suggested that changes within the V1-V2 domain can affect receptor/coreceptor utilization and infection efficiency in different cells (16, 21, 26, 37, 38, 40, 41). Changes in the lengths and/or glycosylation patterns of the V1-V2 loops may impact cell entry by influenc-

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TABLE 1. V1-V2 length and number of PNGS during early versus chronic infection

Subject	Value for indicated infection phase							
	Early				Chronic			
	Infection interval ^a (mo)	No. of sequences ^b	V1-V2 median	PNGS median	Infection interval ^a (mo)	No. of sequences ^b	V1-V2 median	PNGS median
QA203	1	14	61	5	41	11	59	6
QA284	1	15	60	3	47	12	60	6
QA779	2	17	57	4	37	19	57	5
QB424	1	21	61	5	31	12	73.5	6
QB596	6	16	61	5	24	16	62	5
QB670	1	10	71	6	46	12	72.5	6
QC168	1	13	61	6	32	10	61	6
QC449	5	16	77	7	41	16	77	7
QC890	2	16	80	8	42	14	76	7

^a Duration from estimated date of infection to day of sample collection.

^b Number of independent sequences analyzed.

ing the accessibility of the viral envelope receptor-binding domain. Thus, V1-V2 changes associated with neutralizing antibody escape may also alter viral-envelope-cellular-receptor interactions because V1-V2 modifications over the course of infection are likely shaped by the interplay between the need to retain envelope function and to evade the antigenic selection pressure.

In the present study, we compared HIV-1 subtype A V1-V2 envelope sequences isolated early in infection to those isolated around 2 to 3 years into the chronic phase of infection, and we investigated the effects of these genotypic changes on antibody neutralization and the ability to infect cells with various levels of receptors/coreceptors. We found that envelope sequences isolated during the chronic phase of infection had significantly greater numbers of amino acids and potential N-linked glycosylation sites (PNGS) within the V1-V2 loops than viruses early in infection. We created chimeric envelopes by substituting V1-V2 loops from different time points during infection into a parental backbone, and for recombinant viruses, we show that V1-V2 loop changes over the course of a natural infection alter the sensitivity of the viral envelope backbone to antibody neutralization and have a modest effect on infection efficiencies.

MATERIALS AND METHODS

Subjects and envelope sequences. PCR amplification and sequence analysis of the subject's HIV-1 envelope V1-V3 sequences at the two time points during infection have been described previously (34). Eight previously examined subjects (34) and one additional subject (QC449) were selected for this study based on both the availability of viral sequences at the two separate time points and the subtype of the HIV-1 infection (Table 1). All subjects acquired HIV-1 through heterosexual contact (18) and were documented to have subtype A infection (29) (Fig. 1). The method of estimating the date of infection has been previously described in detail (35).

Construction of recombinant HIV-1 virus stocks. All sequences were aligned using Clustal X and further manually codon aligned using MacClade (version 4.01). We defined the V1, V2, and V1 through V2 (V1-V2) regions as amino acids corresponding to HXB2 envelope amino acids 131 to 152, 161 to 196, and 131 to 196, respectively (19). The numbers of amino acids and PNGS in the V1, V2, and V1-V2 portions of each sequence were determined through manual counting and by using the tool at <http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html>, respectively.

Chimeric envelopes were constructed by exchanging subjects' V1-V2 early- and chronic-infection sequences into a parental HIV-1 (Q23) envelope expression plasmid. Q23 is a full-length subtype A clone isolated approximately 1 year

after infection from primary culture (27), and the Q23 envelope (Q23gp160) used here was derived from the full-length clone by subcloning into the pCR3.1 mammalian expression vector (Invitrogen, Carlsbad, CA) (S. Rainwater and J. Overbaugh, submitted for publication). For the majority of subjects, an HpaI-BsmI restriction fragment from the Q23 envelope expression plasmid was replaced by the corresponding fragment from each subject's most commonly amplified envelope sequence at both time points. For QB424, there was no predominant sequence during chronic infection, and from the sequences of this subject, two chimeric envelopes were created, one with the longest V1-V2 and one with the most V1-V2 PNGS. Two chimeric envelopes were created from QA203 chronic-infection sequences, one with the most common V1-V2 sequence and another one with an atypical 11-amino-acid insertion in V1. For two subjects (QB596 and QC168), an HpaI-BglII fragment was exchanged because their PCR-amplified envelope sequences did not have a BsmI site. HpaI cuts upstream of V1 (after nucleotide 6590 in HXB2), and BsmI and BglII cut downstream of V2 (after nucleotides 7175 and 7046 in HXB2, respectively). The translated chimeric envelope proteins incorporate fragments from each subject, from around 8 amino acids prior to the start of V1 to around 16 amino acids into V3 for the BsmI-cut fragments and around 23 amino acids prior to V3 for the BglII-restricted fragments. All chimeric envelope constructs were verified by sequence analysis. Figure S1 in the supplemental material shows all the predicted amino acid differences between the pseudotyped viruses, with early- versus chronic-infection sequences.

The chimeric envelope of interest and either the entire Q23 genome lacking the envelope gene (Q23ΔEnv) (15) or an HIV-1 BRU construct without an envelope gene and with a green fluorescent protein (GFP) reporter gene (BRUΔEnvGFP) (47) were transiently cotransfected into 293T human embryonic kidney fibroblasts by using the Fugene protocol (Roche Molecular Biochemicals) to generate Q23 and BRU-GFP single-cycle-pseudotyped viruses, respectively. For all pseudotyped viruses, 293T cells were transfected with 2.67 μg of a chimeric envelope along with either 5.33 μg of Q23ΔEnv or 5.33 μg of BRUΔEnvGFP. The 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (complete DMEM). The media were replaced after 24 h. After 48 h in culture, the supernatant was filtered through a 0.22-μm filter, aliquoted, and stored at -80°C until use.

The infectious dose of the virus was determined by infecting TZM-bl cells with a range of viral dilutions. TZM-bl is a HeLa-based cell line that has been genetically engineered to stably express high levels of CD4 and CCR5; in addition, TZM-bl cells express *Escherichia coli* β-galactosidase under the transcriptional control of the HIV-1 long terminal repeat (42). The cells were stained for β-galactosidase expression 48 h postinfection, and the number of blue cells was counted to estimate the infection titers as described previously (12).

Neutralization assay. Neutralization assays were performed as described previously using TZM-bl cells (45). The 50% inhibitory concentrations (IC₅₀s) were expressed as the reciprocals of the plasma dilutions required to inhibit infection by 50%. An IC₅₀ of 12.5 was designated for plasmas that failed to achieve at least 50% infection inhibition even at the highest dilution of 1:25. Within each experiment, all infections were performed in triplicate and results shown are averages for two or more independent experiments with viral stocks generated by two separate transfections.

FIG. 1. Phylogram of envelope sequences from the nine subjects described here, sequences from other subjects previously isolated in our laboratory, and reference subtype sequences from the Los Alamos HIV-1 database. The sequences analyzed include nucleotides 340 to 1107 relative to the coding sequence for the HXB2 reference strain gp160 envelope gene. Sequences were aligned using Clustal X and further manually codon aligned using MacClade (version 4.01). After gap stripping, a neighbor joining tree was constructed in the software package Phylogenetic Analysis Using Parsimony and Other Methods (PAUP 4.02b2a) (39) with a general-time-reversible model using a gamma distribution rate parameter of 0.5. For the sake of clarity, only one sequence from a set of identical sequences was included in this phylogram. Early-infection sequences are denoted by black boxes and chronic-infection sequences are denoted by gray boxes. Numbers at the nodes represent the bootstrap values for those nodes from 100 bootstrap resamplings. H.BE.93.VI was used as an outgroup because it was likely to have the least homology with the other sequences. The clusters formed by the subjects' sequences and the HIV-1 subtype grouping are labeled to the right of the trees. Q23, the backbone envelope used for the construction of all chimeric envelopes, is also labeled.

Infection efficiency experiments. HeLa-derived cell lines with different CD4 and CCR5 concentrations were generously provided by Emily Platt and David Kabat (25). All cells were plated at 4×10^4 cells per well in a 24-well dish with complete DMEM. The next day, the medium was removed, and the cells were incubated at 37°C with 1×10^4 BRU-GFP infectious pseudotyped viruses and 20 µg/ml of DEAE dextran in a total of 300 µl complete DMEM. After 2 h of incubation, 0.7 ml of complete DMEM was added. Cells were collected by trypsinization and centrifugation after 48 h postinfection. Cells were incubated in 4% paraformaldehyde in phosphate-buffered saline for 5 min and washed twice with phosphate-buffered saline. The number of infected cells was assessed by

measuring GFP staining by flow cytometry. Cells incubated in complete DMEM alone were used as negative controls. Pseudotyped viruses from cotransfections of BRUΔEnvGFP with a plasmid encoding the vesicular stomatitis virus G envelope (1) were used as positive controls for all cell lines. Within each experiment, all infections were performed in triplicate and results shown are averages for two or more independent experiments with viral stocks generated by two separate transfections.

Statistical analysis. Comparisons of the nine subjects' median values for the numbers of amino acids or PNGS at the two time points were done using the Wilcoxon matched-pair signed-rank test. The Wilcoxon rank sum test was used

to compare distributions within subjects. The Wilcoxon rank sum test stratified by subject was used to compare the aggregate difference between early- and chronic-infection sequences in all subjects. To compare neutralization sensitivity to autologous plasma between the pseudotyped viruses with different groups of V1-V2 sequences, IC_{50} values were dichotomized into values above and below the detection limit of the neutralization assay (IC_{50} of 25). We used generalized estimating equations (GEE) with a logit link and an unstructured correlation structure to create a longitudinal model. Covariates in this model included a group variable (pseudotyped viruses with either the early, the chronic, or the parent [Q23] V1-V2 sequence), duration of time from estimated day of infection to plasma collection date, and an interaction of these two variables. The average IC_{50} s of the pooled plasma against pseudotyped viruses with V1-V2 sequences isolated at different points in infection were compared using the Wilcoxon matched-pair signed-rank test. The average relative infection levels between pseudotyped viruses with V1-V2 sequences isolated at different points in infection were compared using the Wilcoxon matched-pair signed-rank test. All P values are based on a two-sided test. All statistical analyses were done with Intercooled Stata version 8.0 (Stata Corporation, College Station, TX) and SAS version 8.2 (SAS Institute, Cary, NC).

Nucleotide sequence accession numbers. The majority of the sequences reported in this publication were previously assigned GenBank numbers (DQ136320 to DQ136438 and DQ136449 to DQ136465), and the sequences from subject QC449 were newly determined in this study and assigned GenBank numbers DQ376203 to DQ376234.

RESULTS

Subjects and sequences. The V1-V3 sequences examined here were isolated about 1 to 6 months postinfection for the early-infection sample and approximately 24 to 47 months after the estimated dates of infection for the chronic-infection sample (Table 1). The median interval of time between the collection days for the two samples was about 35 months (range, 18 to 46). We compared a median of 16 (range, 10 to 21) and a median of 12 (range, 10 to 19) sequences isolated during early and chronic infection, respectively, from each subject (sequences from all subjects except QC449 were described previously) (34) (Table 1). Each sequence was derived from an independent PCR starting with one template from the subject's peripheral blood mononuclear cells to minimize resampling bias (34). All sequences were aligned with 115 envelope sequences previously isolated from 66 different subjects in our laboratory and 98 reference sequences from the Los Alamos Database. For each subject, sequences at both time points clustered together, suggesting that there was no contamination or evidence of reinfection by two different partners (Fig. 1). Furthermore, sequences from different subjects differed from each other by a minimum of 10% (using a general-time-reversible model with a gamma distribution rate parameter of 0.5) (data not shown).

V1-V2 length and PNGS. The median V1-V2 length in a subject ranged from 57 to 80 amino acids in the early-infection sequences and 57 to 76 amino acids in the chronic-infection sequences (Table 1). The median number of PNGS within the V1-V2 region in a subject ranged from 3 to 8 early in infection compared to 5 to 7 in sequences isolated during the chronic phase of infection (Table 1). There was no significant difference in the median V1-V2 lengths ($P = 0.75$) or the median numbers of PNGS ($P = 0.16$) between early- and chronic-infection sequences by the matched-pair signed-rank test. Because we isolated multiple sequences from the same subject at each time point, we used the rank sum test to assess for changes in these distributions within each subject. Using this test, we observed a significant increase in V1-V2 length in the

chronic-infection sequences for QA779, QB424, QB596, and QB670 (Fig. 2A). For QC890, sequences isolated early in infection had significantly longer V1-V2 lengths than sequences isolated during chronic infection. There was no statistically significant change in V1-V2 length between sequences at the two time points for the remaining four subjects (QA203, QA284, QC168, and QC449). When the data were considered in aggregate, envelope V1-V2 sequences isolated during chronic infection (median, 64; range, 56 to 84) were significantly longer than those isolated during the primary phase of infection (median, 61; range, 57 to 80; $P = 0.02$, stratified rank sum test).

We performed a similar intrasubject analysis for the numbers of PNGS within the V1-V2 domains by using the rank sum test. For six of the nine subjects (QA203, QA284, QA779, QB424, QB596, and QC449), the chronic-infection sequences had significantly greater numbers of PNGS than the early-infection sequences (Fig. 2B). For two subjects (QB670 and QC168), there were no statistically significant changes in the numbers of PNGS between the two time points. One subject (QC890) had a lower distribution of PNGS in the sequences isolated during chronic infection than in the sequences isolated early in infection. Collectively, chronic-infection sequences (median, 6; range, 4 to 8) had significantly higher numbers of PNGS than early-infection sequences (median, 5; range, 3 to 8; $P < 0.001$, stratified rank sum test).

When we analyzed the V1 loop and the V2 loop separately, early-infection V1 envelope sequences (median length, 16 [range, 8 to 24]; median number of PNGS, 2 [range, 1 to 3]) were significantly shorter ($P < 0.001$, stratified rank sum test) and had fewer PNGS ($P = 0.002$, stratified rank sum test) than sequences isolated during chronic infection (median length, 18 [range, 8 to 34]; median number of PNGS, 3 [range, 1 to 5]). V2 sequences isolated early in infection (median length, 38 [range, 34 to 49]; median PNGS, 1 [range, 0 to 3]) showed no significant length change ($P = 0.15$, stratified rank sum test) but had significantly lower numbers of PNGS ($P < 0.001$, stratified rank sum test) than sequences isolated during the chronic phase of infection (median length, 40 [range, 34 to 48]; median number of PNGS, 2 [range, 0 to 3]). To specifically map the PNGS additions over the course of infection, we aligned the V1-V2 amino acid sequences from each subject to the HXB2 sequence (GenBank accession number K03455) and recorded the positions of PNGS according to the HXB2 amino acid sequence (Fig. 3). PNGS additions generally occurred in the N-terminal portion of V1, often in conjunction with insertions/deletions. Within V2, PNGS additions generally occurred in the C-terminal portion as a result of mutations which created the context required for N-linked glycosylation (17).

Infectivity of pseudotyped viruses generated with chimeric envelope sequences. To assess the impact of V1-V2 changes on antibody neutralization sensitivity, we created chimeric envelopes by replacing the V1-V2 domains from an HIV-1 subtype A (Q23) envelope expression plasmid (S. Rainwater and J. Overbaugh, submitted for publication) with sequences of interest. Figure 4 shows the predicted V1-V2 amino acid differences between Q23 and each subject's early- and chronic-infection sequences used to construct the chimeric envelopes. Sequence differences flanking the V1-V2 domains were also incorporated into the chimeric envelopes (see Fig. S1 in the

FIG. 2. Distribution of the number of amino acids (A) and PNGS (B) within V1-V2 loops in sequences isolated during early (black bars) and chronic (white bars) infection. The numbers of amino acids (A) or PNGS (B) are shown along the x axes, and the fractions of the sequences are shown along the y axes. Each subject's graph shows the Z and P values for a Wilcoxon rank sum test comparing the distribution of the V1-V2 lengths (A) or PNGS (B) for sequences isolated early in infection versus that for chronic-infection sequences.

Subject	N-terminal part of V1 (131-141)	C-terminal part of V1 142-152	Position 160	N-terminal part of V2 161-183	C-terminal part of V2 184-196
QA203	ID (1/11) -1	ID (2/11) +2			M (9/11) +1
QA284	ID (9/12) +1				M (12/12) +1 - +3
QA779	M (15/19) -1		M (19/19) +1	M (17/19) +1	
QB424	ID (9/12) +1 - +2	M (1/12) -1		M (11/12) -1	ID (8/12) +1 - +2
QB596	ID (4/16) +1 - +2				M (7/12) +1 - +2
QB670	ID (1/12) -2	M (7/12) -2 - +1			
QC168	ID (5/10) +1	M (4/13) -1			M (1/13) +1
QC449					M (7/16) +1
QC890	M (2/14) +1	M (2/14) -1		M (4/14) -1	ID (6/14) -1

FIG. 3. Changes in the positions of PNGS in the chronic-infection sequences relative to that in the paired predominant early-infection sequences within each subject. The PNGS positions were assigned according to the HXB2 envelope amino acid numbering. Changes in the numbers of PNGS occurred as a result of either insertions/deletions (ID) or mutations (M). The proportions of the chronic-infection sequences with PNGS changes are listed in parentheses. The numbers or ranges for the PNGS differences in the chronic-infection sequences relative to those in the early-infection sequences are shown outside the parentheses.

supplemental material). For seven subjects, the early- and chronic-infection C2 and V3 envelope regions incorporated into the chimeric envelopes did not have any length variation or difference in number or position of N-linked glycosylation sites. For QA284, the early- and chronic-infection C2-V3 regions incorporated into the chimeric envelope had different numbers and sites of potential glycosylation. For QB670, the chronic-infection C2-V3 region incorporated into the chimeric envelope had two more PNGS than the early-infection sequence. All of the envelope chimeras, except one (QA779), were functional as judged by their ability to pseudotype Q23ΔEnv, which is the Q23 subtype A virus with a nonfunctional envelope gene (15).

The median titer of the pseudotyped viruses generated with these envelope chimeras was around 6.3×10^5 infectious particles (IP)/ml (range, 1.5×10^5 to 2.1×10^6 IP/ml), while the approximate titer for Q23ΔEnv pseudotyped with its own envelope was around 2.2×10^5 IP/ml. Pseudotyped virus with QA779 early-infection envelope sequences produced large amounts of p24 but no discernible infection titer (data not shown), and thus, no further functional studies were performed with the QA779 envelope sequences from either time point. Unlike all the other variants examined in this study, QA779 variants isolated early in infection did not have a predicted glycosylated asparagine at the base of envelope variable loop 2 (corresponding to HXB2 amino acid

	131	140	V1	160	180	V2	196								
HXB2	CTDLK	NDTNT	NSSSGRMIMEK	G	EIKNCSP	NI	STSI	RGKVQKEYAFFYKLDIIPID	NDTTS	YKLTSC					
Q23	-----NV	SVNTTD	-----	EGL-N	---	NMT-EL-D-R-V-SL	---	V-NE	NQG	SE	-----R-IN				
QA203D (M1)	-NTA	..NF	-----NNVTNDM	E	---	NMT-EL-D-K-V-SL	---	VV-F	..N	..N	..SRQ	-----R-IN			
QA203A (M41)	-SNVTNV	---	-----NDM	R	---	NMT-EL-D-K-V-SL	---	VV-F	..N	..N	..SSQ	-----R-IN			
QA203B (M41)	-SNV	..NV	---NVNVTVNNVNTNVNTNDM	T	---	NMT-EL-D-K-V-SL	---	VV-F	..N	..N	..SSQ	-----R-IN			
QA284A (M1)	-HH	..NV	KITQEMK	E	---	NVT-EL-D-K-V-SL	---	VV-F	..NS	..DDSSP	-----R-IN				
QA284A (M47)	-H-CHHNV	NITQDM	T	---	NVT-EL-D-K-V-SL	---	VV-LK	..NS	..DNNSS	-----R-IN					
QA779A (M2)	-STVN	..NSS	-----	E	---	N-TY	---A-EL-D-K-VNSL	---	VVQ	NERK	..NSN	..Q	-----R-IN		
QA779F (M37)	-STVT	..NSS	-----	E	---	N-TYN	---T-EL-D-K-V-SL	---	VVQ	NE	..SENS	..N	..Q	-----R-IN	
QB424B (M1)	-SNV	..NV	GNKV	NIT	---R-EDM	---	N-T-E	---D-KKQA-SL	---	V-SS	..NSS	NE	-----R-IN		
QB424D (M31)	-SNV	..NV	DRDVNTVSNVTVSKDTVTDNKGNN	---W	EDM	---	N-T-E	---D-KKQA-SL	---	V-SST	..NNNS	NE	-----R-IN		
QB424L (M31)	-S	NV	VTGNRTGNEGNNNTGGENM	..KNT	---G-EDM	---	N-T-E	---D-KKQA-SL	---	VV-SS	..NNS	NE	-----I-IN		
QB596F (M6)	---NV	..NS	GN	STESMQ	E	MRN	---	NMT-EL-D-K-V-SL	---	VV	-----SNNNS	..E	-----R-IN		
QB596M (M24)	---NVSSNI	GNSTENITGNSTENMR	E	MRN	---	NMT-E	---D-TK-V-SL	---	VV	-----SNNNS	..E	-----R-IN			
QB670E (M1)	-S	NV	RNS-V	..TGNN	---TVEA	E	---	NMT-EL-D-KK-V-SL	---	VVQ	---QGNSSSSS	..NN	..E	-----R-VN	
QB670B (M46)	-SNV	..NV	RNN	---	..TGNDTV	---A	E	---	NMT-EL-D-TK-V-SL	---	VVQ	---QGNSSSSS	..NN	..E	-----R-VN
QC168E (M1)	---NVN	..NN	---T	VNNT	WDE	---R-N	---	NVT-EL-D-R-V-SL	---	VVQ	-----NSS	-----R-IN			
QC168F (M32)	---V	..NN	---VN	..N	..TTNGNNSM	WDE	---R-N	---	N-T-E	---D-R-V-SL	---	VVQ	-----NSS	-----R-IN	
QC449A (M5)	---NVNVTRNA	SN-DNATHVINK	D	---	N	---	N-T-E	---KD-KK-A-SL	---	R-VQ	---EKQGNSSN	---SYND	-----R-IN		
QC449A (M41)	---NVNVTRNA	SN-DNATHVINK	D	---	N	---	N-T-E	---KD-KK-A-SL	---	R-VQ	---EKQGNSSN	---SYKD	-----R-IN		
QC890 (M2)	---NVT	..NG	---SEGNTFTVSEDMT	-----	N	---	NMT-EL-D-QR-V-SL	---	VV-L	DNLNNSSAKLDN	SDSRT	---R-IN			
QC890 (M42)	---NVT	..TGN	---SEGIKFN	VS	---K-MT	E	---	NMT-EL-D-RR-V-SL	---	T-VV-L	DNLNNSSA	LD	-----SRT	---R-IN	

FIG. 4. Subjects' predicted early- and chronic-infection V1-V2 amino acid sequences used to create chimeric envelopes in the Q23 backbone. Each sequence is aligned to the HXB2 reference sequence, shown in single-letter amino acid code. The parent Q23 V1-V2 sequence is shown below the HXB2 reference. The name of each sequence identifies the subject and the time, in number of months after estimated date of infection, when the V1-V2 sequences were isolated. Relative to the HXB2 sequence, a dash indicates no amino acid change and a dot indicates an insertion or deletion. All PNGS are identified in bold. The V1 and V2 domains and the HXB2 envelope amino acid number are indicated above the HXB2 sequence.

mon subtype A Q23 HIV-1 backbone. To evaluate the effect of subjects' early- and chronic-infection V1-V2 sequences, we first determined whether each subject's longitudinal plasma contained antibodies that could neutralize the parent Q23 envelope. The Q23 envelope sequence is not closely related to

FIG. 6. Neutralization of pseudotyped viruses with pooled plasma. Plasma pools were collected by combining plasma from 30 different HIV-1-infected Kenyan subjects. The virus names on the *x* axis identify the subjects and the times, in number of months after estimated date of infection, when the V1-V2 sequences were isolated. The *y* axis shows the IC₅₀s (the reciprocals of the plasma dilutions required to inhibit infection by 50%). All IC₅₀s represent mean values from two or more independent experiments with viral stocks from two separate preparations. The error bars show the 95% confidence intervals. QC168(gp160) denotes a pseudotyped virus with a full-length early-infection envelope. Q23 denotes the parent Q23 virus pseudotyped with an envelope carrying the native V1-V2 loops from the original clone.

any of the other subjects' sequences (Fig. 1). The genetic distances between the subjects' sequences and Q23 ranged from approximately 10% to 25% with a general-time-reversible evolutionary model using a gamma distribution rate parameter of 0.5 (data not shown). In addition, the V1-V2 sequence of Q23 consists of 59 amino acids with four PNGS (Fig. 4). For the majority of subjects, the heterologous plasma did not neutralize the Q23 virus pseudotyped with its own envelope at various times after infection (Fig. 5). For two subjects (QA284 and QC890), only plasma collected late in infection neutralized the Q23 envelope. For two other subjects (QB424 and QC449), plasma showed neutralizing activity against the Q23 envelope that was marginally higher than the designated assay cutoff. Thus, the parental virus was neutralization resistant to the majority of the subjects' plasma at various times after infection.

We next evaluated the neutralizing activity of the same longitudinal plasma samples against pseudotyped viruses with chimeric envelopes incorporating autologous V1-V2 domains in the Q23 envelope. Introduction of early-infection V1-V2 sequences into the parental virus rendered the Q23 backbone pseudotyped virus significantly more sensitive to plasma neutralization ($P = 0.02$, GEE logistic regression) than the Q23 pseudotyped virus with its native V1-V2 loops. In the multiple-logistic-regression GEE model, time postinfection for the plasma sample was also a significant predictor of neutralization IC₅₀ ($P = 0.03$). Thus, plasma samples collected later in infection displayed significantly greater neutralizing activity against the pseudotyped Q23 virus with early-infection V1-V2 loops. This is well illustrated with plasma samples collected >6 months after V1-V2 sequences were isolated for six of the

eight subjects (QA203, QA284, QB424, QB596, QB670, and QC168) (Fig. 5). Neutralization activity was detected against all pseudotyped viruses with early-infection V1-V2 loops, although in some subjects (QA203, QC449, and QC890), it was observed more than 3 years after the isolation of the early-infection sequences. For these subjects, neutralizing antibodies could not be detected in plasma around 1 year after the V1-V2 sequences were isolated, and intervening samples were not available to more precisely define when these antibodies first arose.

Pseudotyped viruses with chronic-infection V1-V2 sequences were not more sensitive to plasma neutralization than the parent Q23 virus ($P = 0.36$, GEE logistic regression). Similar to viruses with early-infection V1-V2 sequences, however, pseudotyped viruses with chronic-infection V1-V2 loops showed evidence of neutralization sensitivity to plasma samples collected >6 months after the sequences were isolated. However, only a limited number of plasma samples were available beyond the collection day for the chronic-infection sequences. For three subjects (QA284, QB670, and QC890), plasma samples were available 6 months beyond the collection day for the chronic-infection V1-V2 sequences. QA284 and QC890 pseudotyped viruses with chronic-infection V1-V2 loops were neutralized by the autologous plasma samples from late in the infection (Fig. 5). For these two subjects, however, we cannot rule out that neutralization may have been directed against Q23 sequences and not related to introduction of the subject's V1-V2 sequences because plasma samples also showed activity against the heterologous Q23 virus with its native V1-V2 loops. Because we did not examine many plasma samples collected >6 months after the time when chronic-

FIG. 7. Relative infection levels for the low-CD4, medium-CCR5 (RC-49) (A) and high-CD4, low-CCR5 (JC-10) (B) cell lines (25) with recombinant pseudotyped viruses with chimeric envelopes. The virus names on the x axes identify the subjects and the times, in number of months after estimated date of infection, when the V1-V2 sequences were isolated. The y axes show the infection levels for the respective cell lines. These values are normalized relative to the infection levels for the high-CD4, high-CCR5 (JC-53) cell line (25). All infection levels represent mean values from two or more independent experiments with viral stocks from two separate preparations. The error bars show the 95% confidence intervals.

infection sequences were isolated, we could not assess whether de novo responses that would neutralize the chimeric viruses developed in these individuals.

Pseudotyped viruses with early-infection V1-V2 loops were significantly more sensitive to plasma neutralization than the recombinant viruses with chronic-infection V1-V2 sequences ($P < 0.001$, GEE logistic regression). This was especially true for plasma samples collected later in infection because number of months postinfection for the plasma sample was a significant predictor of the IC_{50} ($P = 0.02$, GEE logistic regression). Thus, isogenic viruses with different V1-V2 loops displayed differential antibody neutralization sensitivities to plasma samples isolated >6 months after infection.

To further assess neutralization sensitivity differences between early- and chronic-infection pseudotyped viruses, we compared neutralization sensitivity against heterologous pooled plasma. We generated plasma pools by combining plasma from 30 different HIV-1-infected Kenyan individuals

distinct from the 9 subjects examined in this study. The pooled plasma neutralized all pseudotyped viruses, with a median IC_{50} of 166.59 (range, 86.98 to 378.79) (Fig. 6). For the majority of subjects, the differences in IC_{50} s between the early- and chronic-infection viruses were relatively small. As a group, pseudotyped viruses with early-infection V1-V2 sequences (median IC_{50} , 142.53; range, 86.98 to 256.94) were not significantly more sensitive to neutralization by heterologous pooled plasma than the group of viruses with chronic-infection V1-V2 loops (median IC_{50} , 166.58; range, 104.97 to 234.62; $P = 0.33$, matched-pair signed-rank test).

For QC168, we also isolated a full-length envelope (gp160) from a peripheral-blood-mononuclear-cell sample 1 month after the estimated date of infection, similar to the isolation of the early-infection QC168 V1-V2 sequences (Table 1). Viral stocks of this full-length envelope pseudotyped with Q23ΔEnv had an approximate infection titer of 2.6×10^6 IP/ml. QC168 longitudinal plasma samples showed neutralizing activity

against this pseudotyped virus around 5 months after the collection day for the early-infection viral envelope sequences (Fig. 5). Neutralizing activity against the pseudotyped virus with the full-length gp160 was considerably higher and was observed earlier than that against pseudotyped virus with QC168 early-infection V1-V2 sequences. The full-length QC168 envelope virus, however, demonstrated a level of sensitivity to heterologous antibodies relatively similar to that of the pseudotyped virus with early-infection QC168 V1-V2 segments alone. (Fig. 6)

Cell infection. To assess the impact of V1-V2 changes on viral entry, we created pseudotyped viruses with the chimeric envelopes and the BRUΔEnvGFP construct (47) and examined their infectivities in cell lines expressing various amounts of CD4 and CCR5 (25). Viral infectivity was highest for cells expressing the largest amounts of both receptors (JC-53). Thus, the level of infectivity in the JC53 cells was used as a reference of 100%. The infection levels in the RC-49 (low levels of CD4 and medium levels of CCR5) and JC-10 (high levels of CD4 and low levels of CCR5) cell lines ranged from approximately 5 to 50% relative to those in the JC-53 cell line (Fig. 7). For the majority of subjects, pseudotyped viruses infected the low-CD4 cell line with lower efficiency than the low-CCR5 cell line.

Although the relative infection differences were small, viruses with V1-V2 loops isolated early in infection (median, 16.0; range, 5.0 to 24.0) showed a trend in infecting the low-CD4 cell line with higher efficiency than viruses with V1-V2 sequences isolated during the chronic phase of infection (median, 10.2; range, 7.3 to 20.1; $P = 0.07$, matched-pair signed-rank test). For six of the eight subjects (QA203, QA284, QB424, QB670, QC449, and QC890), pseudotyped viruses with V1-V2 loops isolated early in infection were better at infecting the low-CD4 cell line than the viruses with chronic-phase V1-V2 sequences (Fig. 7A). For the remaining two subjects (QB596 and QC168), viruses with early-infection V1-V2 loops were less efficient at infecting the low-CD4 cell line than viruses with V1-V2 regions isolated during the chronic phase of infection.

Pseudotyped viruses with V1-V2 sequences isolated during chronic infection were better at infecting the low-CCR5 cells in six of eight subjects (QA203, QA284, QB424, QB670, QC168, and QC449) and marginally worse in the other two subjects (QB596 and QC890) than viruses with V1-V2 sequences isolated early in infection (Fig. 7B). Although the differences in the relative infections were small, there was a trend that viruses with V1-V2 sequences isolated during the chronic phase of infection (median, 27.1; range, 22.4 to 40.1) were more efficient at infecting cells with low CCR5 concentrations than viruses with V1-V2 sequences isolated early in infection (median, 24.4; range, 5.2 to 33.4; $P = 0.09$, matched-pair signed-rank test).

DISCUSSION

This study of nine subjects with HIV-1 subtype A infection indicates that increased glycosylation sites and/or shifts in their position in the V1-V2 domain of the envelope glycoprotein contribute to viral adaptation over the course of infection. These findings complement and extend previous results on a small number of subtype B HIV-1-infected subjects ($n = 3$)

(43) and SHIV-infected macaques ($n = 1$) (5). Similar to a previous study (37), we also show that envelope variable loop expansion occurs often over time within a host. These genotypic changes, which occur over the course of a natural infection, impact sensitivity to antibody neutralization. In particular, the early-infection V1-V2 sequences rendered a relatively neutralization-resistant backbone sensitive to neutralizing antibodies present several months to years later within the infected individual. However, the same antibodies generally did not neutralize viruses bearing chimeric envelopes carrying V1-V2 sequences that evolved during chronic infection. Thus, V1-V2 expansion and increased glycosylation may represent a general mechanism that HIV-1 subtype A uses to evade the host responses. Similar to a previous study of envelope sequence changes in tissue culture (28), our observations from HIV-1 variants isolated over the course of infection suggest that changes within the V1-V2 domains may be constrained by the effect that they have on envelope function; there may be subtle effects of V1-V2 changes on receptor/coreceptor requirements, although our findings were not statistically significant.

By analyzing a number of subjects collectively, we determined general features about V1-V2 changes over the course of infection. V1 PNGS modifications predominantly occurred in conjunction with insertions/deletions at the N segment of the loop, and the majority of V2 PNGS additions resulted from mutations in the C-terminal portion of the loop (Fig. 3). This pattern of PNGS differences between early- and chronic-infection envelope sequences is similar to that observed between early-infection sequences from newly infected infants and chronic-infection sequences from the transmitting mothers (45). In addition, macaques infected with a cloned SHIV also displayed similar types of glycosylation changes over the course of infection (2). Results from these diverse viruses in these various hosts imply that glycosylation patterns follow a consistent and predictable course during infection. Furthermore, our results suggest that both V1 and V2 loops can accommodate alterations in glycosylation, but the V1 loops are more likely to have length changes. Expansion and increased glycosylation within the V1-V2 segments over the course of infection, however, were not observed in all subjects in this study or in all subjects in other published studies (37, 43). This is exemplified by QC890, for whom variants present during chronic infection had significantly shorter variable loops with fewer PNGS than variants isolated early in infection. Thus, there are also likely sequence solutions other than those examined here that allow viruses to persist in the face of humoral immune responses.

Although PNGS additions are a general feature of HIV-1 evolution, it remains to be determined whether the new PNGS are truly glycosylated. We evaluated potential glycosylation by counting asparagines (N) in an N-X-serine (S) or -threonine (T) pattern, where X can be any amino acid except proline. Certain amino acids may prevent all asparagines in this context from being glycosylated (11, 17, 36). For instance, numerous variants in a number of subjects showed an NNSS or NNTT amino acid pattern (Fig. 4). In our analysis, we counted two PNGS with these patterns, but steric hindrance may prevent carbohydrate addition to both asparagines. When we counted one PNGS with an NNSS or NNTT amino acid pattern, chronic-infection sequences still had a significantly higher number of

PNGS than the early-infection sequences (data not shown). Although steric hindrance may prevent multiple adjoining asparagines from being glycosylated, this pattern may allow glycosylation sites to be easily shifted.

Our studies suggest that shifts in the glycosylation sites and other V1-V2 changes over the course of infection likely occur because of selective pressure from the host immune response, and the need to retain envelope function limits the extent of envelope modifications. Sequence differences flanking the V1-V2 loops incorporated in the chimeric envelopes may have also contributed to differences we observed in antibody neutralization and infectivity. The majority of the sequence changes between the early- and chronic-infection envelope sequences incorporated into the chimeric envelopes, however, were within the V1-V2 domains, and therefore, modifications in the V1-V2 loops are likely to have contributed to the observed phenotypic differences.

We showed that introducing V1-V2 loops from different subjects at various times after infection into a common viral backbone changed the neutralization sensitivity of the recombinant pseudotyped virus to autologous plasma. These observations confirm the conclusions from previous studies that V1-V2 loops influence susceptibility to antibody neutralization (24). The majority of a subject's autologous plasma samples did not neutralize pseudotyped viruses with that subject's chronic-infection V1-V2 sequences. This was perhaps not surprising because the chronic-infection V1-V2 sequences were isolated at a later time in infection than the autologous plasma sample tested. The differences in neutralization susceptibility between chimeras with early- and chronic-infection V1-V2 sequences support the notion that modifications in the V1-V2 loops over the course of an infection contribute to neutralization escape. That V1-V2 domains from different times in infection have different effects on a common resistant viral backbone implies that there may be structural differences in the V1-V2 loops isolated early in infection compared to those isolated during the chronic phase of infection. Neutralizing antibodies may directly target epitopes on the V1-V2 loops (9, 49). Over time within a host, sequence changes in the V1-V2 domains may allow viruses to escape these V1-V2-directed antibodies. Another biological mechanism may also explain our observations. V1-V2 expansion and/or glycosylation modifications over the course of infection may change the structure of the variable loops in a manner that may more effectively shield conserved neutralization-sensitive epitopes on the viral envelope glycoprotein. In support of this model, numerous studies suggest that V1-V2 loops shield conserved neutralization-sensitive regions in the envelope glycoprotein, such as the receptor-binding site (7, 24, 30, 46). In addition, a previous study has demonstrated that amino acid changes in the V1 sequence itself do not impact antigenicity; only V1 amino acid changes that create a glycosylation site impart neutralization resistance. This suggests that V1 carbohydrates function by shielding antibody epitopes (4). More-detailed studies will be required to distinguish whether V1-V2 changes over the course of infection alter sequences of key antibody epitopes and/or lead to conformational masking.

As opposed to the greater sensitivity to autologous plasma, pseudotyped viruses with early-infection V1-V2 loops were not significantly more sensitive to heterologous plasma neutraliza-

tion than viruses with chronic-infection V1-V2 loops. These findings suggest that as the envelope V1-V2 sequences evolve to escape specific antibody pressure within the host, these selected changes do not necessarily render the envelope glycoprotein more resistant to other antibodies. It is important to emphasize that our studies do not address whether viruses present early in infection are more sensitive to neutralizing antibodies than late-stage viruses, because we examined only partial envelope sequences in the context of a common envelope backbone. Thus, our observations do not necessarily conflict with the results from a previous study showing that viruses found in newly infected subjects may be more sensitive to antibody neutralization than the majority of viruses present in the transmitting partners (8). However, we have observed that full-length subtype A envelopes isolated early in infection show considerable variation in their sensitivity to the same pooled plasma tested in this study, with several being poorly neutralized (C. Blish and J. Overbaugh, submitted for publication). Thus, further studies with full-length envelopes are needed to determine whether viruses from early stages of HIV-1 infection differ in their neutralization sensitivities compared to later-stage viruses.

For one subject (QC168) for whom we were able to test neutralization against a full-length envelope (gp160) isolated early in infection, we observed high neutralizing titers within about 5 months after infection (Fig. 5). The relatively rapid appearance of neutralizing antibodies against the pseudotyped virus with a full-length envelope is similar to what has been observed in previous studies (31, 33, 43). This may suggest that the cumulative effect of antibodies against different portions of the envelope protein may be greater than that against the V1-V2 domains alone. On the other hand, it is also possible that V1-V2 antibodies or conformational epitopes that depend on the V1-V2 loops may appear later in infection.

Structural changes of the V1-V2 loops are constrained by the need to retain envelope function. Our results suggest that changes within the V1-V2 envelope loops may have a small negative impact on viral entry efficiency in cells with low CD4 concentrations but slightly improve the ability to infect cells with low CCR5 concentrations. Although the differences in infection efficiencies were small, they may be relevant for some cell types. The levels of cell surface receptors differ among different mononuclear cell types and depend on the body compartment and exogenous factors (44, 48). For instance, macrophages have been shown to have relatively low concentrations of CD4 receptors (14, 22), and thus, the RC49 cell line may be similar to macrophages. It is possible that the difference in infection efficiency between the two groups of viruses may be magnified with multiple rounds of infection. Therefore, replication-competent viruses with envelope variable loops from either the early or the chronic phase of infection may have differential replication kinetics in different cells from various anatomic sites. In addition, other sequence modifications within the envelope surface unit and outside the V1-V2 loops may also contribute to replication differences between early- and chronic-infection viruses. While our results are not conclusive, partly due to the assay and the small sample size, they do suggest that the CD4 and CCR5 requirements for envelopes from different phases of infection may warrant further study.

Studies examining the signature genotypic characteristics of variants isolated early in infection perhaps provide the best evidence that changes within the variable loops influence viral infectivity. Previous studies have shown that viral variants with condensed and less glycosylated variable loops are favored for transmission during both heterosexual subtype A and subtype C transmission (6, 8). Variants with lower numbers of glycosylated residues are also favored during mother-to-child transmission of subtypes A, C, and D (45). Here, we show that once these transmitted strains are established, viruses evolve to add additional glycosylation sites and extend variable loop length during the first few years after infection, which contributes to neutralization escape. Thus, collectively, these studies suggest that envelope loop features may come full circle during a cycle of transmission and infection within a host because at transmission, virus envelope loops are restored to a more condensed and less glycosylated form. The selection of variants with shorter and less glycosylated envelope loops at transmission suggests that the envelope modifications that occur over the course of an infection accrue costs in terms of fitness for transmission. Further studies are needed to determine the effects of changes within the viral envelope glycoprotein over the course of infection on envelope function and viral replication.

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