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High Incidence of Unusual Cysteine Variants in gp120 Envelope Proteins from Early HIV Type 1 Infections from a Phase 3 Vaccine Efficacy Trial

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ABSTRACT

During the course of a large-scale HIV-1 vaccine field trial (VAX004), full-length gp120 sequences were determined for 349 new HIV-1 infections. The data collected represent the largest survey of full-length gp120 sequences from new HIV-1 infections ever assembled. Previous studies have shown that subtype B viruses typically possess 18 cysteine residues that are covalently linked to form 9 conserved disulfide bridges. However, in this study we found that approximately 20% of the trial participants possessed envelope proteins with an unusual number of cysteine residues that could very likely result in unusual protein structures. One class of variants included envelope proteins with two additional cysteine residues in close proximity, potentially yielding additional disulfide-bonded loops. Other classes of variants included envelope proteins where amino acid replacements increased or decreased the number of cysteine residues by one, resulting in molecules with either 19 or 17 cysteines, respectively. Initial functional analysis demonstrated that envelope proteins with 19 cysteine residues bind to CD4 and the CCR5 chemokine coreceptor, and are infectious. These results suggest that the protein structure of gp120 in newly transmitted viruses may be more heterogeneous than previously appreciated and potentially represent a new mechanism of virus variation. The disulfide variation that we report here may have important implications for HIV vaccine and drug development efforts.

INTRODUCTION

THE HIV-1 ENVELOPE GLYCOPROTEIN, gp120, is a peripheral membrane protein of approximately 500 amino acids that is located on the surface of the virions and virus-infected cells. The envelope protein mediates attachment and fusion to uninfected target cells via a series of events initiated by binding to at least two specific cell surface receptors including CD4 and one of several chemokine receptors (usually CCR5 or CXCR4).[1] Viral sequence variation in gp120 is thought to be one of the greatest challenges in HIV-1 vaccine development. To date, sequence variation has been studied primarily with respect to the evolution of virus subtypes or clades. Impartial phylogenetic analysis of the HIV-1 genome has defined three large groups (M, N, and O) with at least eight distinct group M virus subtypes (A to G) and numerous interclade recombinants.[2] Significant variation occurs, however, within clades (intraclade variation) that may have important implications for vaccine development and drug design. Variation affecting the binding of neutralizing antibodies has been described for the third variable (V3) domain that determines chemokine receptor usage and syncytia or nonsyncytia-inducing phenotypes,[3,4] in glycosylation patterns, and in residues that are associated with CD4 binding.[5,6] In this report, we describe an additional type of virus variation, variation in sulfhydryl content, that could affect the structure of the HIV gp120 envelope protein and, in principle, may affect antigenic structure, infectivity, or viral fitness. Typically, gp120 possesses 18 cysteine residues that form 9 covalent disulfide bridges. These disulfide bonds create a conserved series of simple and complex disulfide loops,[7] which in some cases define the boundaries of the five variable (V1–V5) and five conserved (C1–C5) domains of gp120.[8] With one notable exception, the number and relative position of disulfide bonds that occur in gp120 are conserved across clades. However, viruses from the subtype AE clade (a circulating recombinant form designated CRF01_AE), found primarily in Southeast

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Asia, typically possess an extra pair of cysteine residues that leads to the creation of an additional disulfide loop in the V4 domain[9,10] (see also Los Alamos HIV Sequence Data Base, http://www.hiv.lanl.gov/content/hiv-db/mainpage.html). Within subtype B viruses, polymorphism in the disulfide structure of the V1 domain was noted in two cases.[11,12] However, this type of variation appeared unusual and was not noted at a high frequency in the thousands of gp120 sequences collected in various gp120 sequence compendia (e.g., Los Alamos HIV Sequence Database). Thus variation in HIV-1 disulfide structure of subtype B viruses has been viewed as exceedingly rare and not contributing in a meaningful way to overall virus variation.

MATERIALS AND METHODS

Sample collection and trial design

We collected gp120 sequences from 349 new infections that occurred over a 4-year period (1998–2002) in connection with a large Phase 3 clinical trial of a candidate HIV-1 vaccine (AIDSVAX B/B). The AIDSVAX B/B trial consisted of ~5000 men who have sex with men (MSMs) and ~300 women considered high risk for acquisition of HIV via heterosexual transmission. The study was designed to test the efficacy of a bivalent gp120 vaccine consisting of alum-adjuvanted MN (CXCR4 tropic) and GNE8 (CCR5 tropic) immunogens. Participants were enrolled in a 2:1 ratio of vaccinee:placebo and followed for up to 2 years postinfection.

HIV-1 gp120 viral RNA preparation and RT-PCR

Viral RNA was isolated from 0.5–1.0 ml of frozen plasma using the ViroSeq Sample Preparation Kit (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions. Full-length gp120 sequences were amplified from plasma samples using reverse-transcription polymerase chain reaction (RT-PCR) at the earliest postseroconversion timepoint. Random hexamers were utilized for the RT reaction followed by nested PCR reactions with the first round primers ED3/ED14 (ED3 5′-TTA GGC ATC TCC TAT GGC AGG AAG AAG CGG-3′ and ED14 5′-TCT TGC CTG GAG CTG TTT GAT GCC CCA GAC-3′) and then the second round primers envB/ED12 (envB 5′-AGA AAG AGC AGA AGA CAG TGG CAA TGA-3′ and ED12 5′-AGT GCT TCC TGC TGC TCC CAA GAA CCC AAG-3′). The RT reaction condition was 37°C for 60 min with a second step of 90°C for 5 min (First Strand cDNA Synthesis kit, Amersham Biosciences, Piscataway, NJ). The PCR thermal cycling conditions for both rounds of PCR started with an initial denaturing step at 94°C for 2 min, then 30 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 3 min, with a final 72°C for12 min (60 cycles total). Taq DNA polymerase and buffer components were supplied by Sigma-Aldrich (St. Louis, MO).

Cloning and sequencing of PCR products

All resulting PCR reaction products were cloned into a bacterial plasmid (pCR 2.1-TOPO; Invitrogen, Carlsbad, CA) using the manufacturer's recommended protocol and sequenced using BigDye 3.1 reaction mix and an ABI-3100 automated DNA sequencer (Applied Biosystems, Foster City, CA). Eight sequencing primers were used to provide coverage of both strands of the gp120 sequence: M13F 5′-GTAAAACGACGGCCAG-3′; BSEQF1 5′-ACCACTCTATTTTGTGCATCA-3′; BSEQF2 5′-AAACTGCTCTTTCAATGTCACCACA-3′; ES7 5′-CTGTTAAATGGCAGTCTAGC-3′; BSEQF4 5′-TCAAATATTACAGGGCTGCTATTAACAAG-3′; BSEQR2 5′-GTTGTATTACAGTAGAAAAATTCCCCTC-3′; BSEQR3 5′-CTGCCATTTAACAGCAGTTGAGTTG-3′; and M13R 5′-CAGGAAACAGCTATGAC-3′.

Virus infectivity studies

To assess the functionality of cloned envelope genes, a pseudotype infectivity assay similar to that described by Richman *et al.*[13] was used. Briefly, amplified envelope sequences were incorporated into an expression vector (pCXAS) using conventional cloning methods (Monogram Biosciences, S. San Francisco, CA). Expression vectors were prepared from single isolated molecular clones. Recombinant HIV-1 stocks expressing patient virus envelope proteins were prepared by cotransfecting HEK293 cells with a defective HIV-1 genomic viral vector lacking the HIV-1 envelope protein and a second expression vector containing the HIV-1 envelope protein of interest. The HIV-1 genomic vector is replication defective and contains a luciferase expression cassette within a deleted region of the HIV-1 envelope gene. Pseudotype viruses were prepared with patient virus envelope proteins as well as CXCR4 and CCR5-dependent control viruses (NL4–3, JRCSF) and the specificity control, amphotropic murine leukemia virus (A-MLV). U87 cells that express CD4/CCR5 and cells that express CD4/CXCR4 were inoculated with the pseudovirus preparations and infectivity was determined 72 h postinoculation by measuring the amount of luciferase activity expressed in infected cells (reported as relative light units or RLUs).

Statistical methods

Statistical comparisons were made using Fisher's exact test for binomial proportions as implemented in StatXact software, version 5.0.3 (CYTEL Software Corporation, Cambridge, MA).

RESULTS

Sequence analysis of the earliest HIV-positive clinical specimens showed that 281 of the 349 infected individuals had viruses that possessed gp120 protein sequences with 18 cysteines typical of most virus subtypes. However, 68 of 349 subjects (19.5%) possessed viruses with unusual numbers of cysteine residues. The frequency of these cysteine variants did not vary between the vaccine and placebo groups and matched the 2:1 ratio of vaccine to placebo recipients in the clinical trial. Of the 68 viruses, 22 possessed two extra cysteine residues for a total of 20 cysteines, 29 subjects lacked a residue for a total of 17 cysteines, 15 possessed an additional residue for a total of 19 cysteine residues, and two subjects lacked two residues for a total complement of 16 cysteine residues (Table 1). Given that the N-terminal region (as defined arbitrarily by residues 1–250) of gp120 contains 12 of the typical molecule's 18 cys-

Table 1. Summary of Viral gp120 Envelope Proteins with Unusual Disulfide Structure[a]

No. cysteines	*Number of trial participants with variants*	*Placebo*	*Vaccine*	*Location of mutations*
16	2	0	2	C1, C1/V1, C2
17	29	11	18	C1, C2, C4, C1/V1, V1/V2, V2/C2, C2/V3, V3/C3, V4/C4
19	15	5	10	C1, C2, C4, C5, V1, V2, V4
20	22	6	16	V1, V2, V4, V5
Totals	68	22	46	

[a]The cysteine mutations occur in an ~2:1 ratio to match the clinical trial randomization of vaccinees:placebos.

teine residues, about two-thirds of the cysteine deletions should be expected in the N-terminal region if deletions occurred at random. Of the 29 single residue deletions, 22, or 76%, were observed in the N-terminal half of the molecule; however, this was not significantly different from the expected percentage of 67% ($p = 0.40$). Insertions, on the other hand, might be expected to occur equally in either half of the molecule if they occurred at random, but this is not what was observed. In particular, 20 out of 22 double insertions occurred in the N-terminal region ($p < 0.01$). Single insertions were also observed more frequently in the N-terminal half of the molecule (10 out of 15), but the difference was not statistically significant ($p = 0.30$). Of the 22 viruses that contained two additional cysteines, both of these additional residues were located in close proximity to each other within the same domain. Moreover, the location of the additional cysteine residues was nonrandom ($p < 0.01$) and highly constrained (Fig. 1). Thus, 19 of the viruses had two additional cysteine residues inserted in the V1 domain where insertions of pairs of cysteine residues in HIV-1 have been reported previously.[11,12] Furthermore, the V1 domain of HIV-2 ROD and the equivalent domain in SIV-MM142 viruses also contain an extra disulfide bridge.[14] Although it has never been proven by peptide mapping, it has been proposed that the extra cysteine residues actually pair with each other to form an additional disulfide bond, to yield the "oven mitt" type of structures shown in Fig. 2A. However, it is theoretically possible that the extra cysteines could disrupt the normal disulfide structures creating additional "lariat" disulfide variants of the type shown in Fig. 2B. Interestingly, most of the "oven mitt" structures are formed in a way so as to include an N-linked glycosylation site in the base of the loop. The type of structure formed may be determined by the size of the new loop created. The sizes of the loops formed in the "oven mitt" structures in this study range from 3 to 23 amino acids in length.

The V1 domain of gp120 is known to be a target of neutralizing antibodies[15,16] and is thought to provide a "cloaking" function by preventing antibodies from having direct access to neutralizing epitopes in the V3 region and CD4 binding site.[17,18] In addition, several studies have shown that the V1 and V2 domains of gp120 can be deleted without interfering with virus infectivity[19–21] and that these viruses are more easily neutralized than wild-type viruses. Thus, these regions do not appear to be necessary for receptor binding or cell fusion. Therefore, it is plausible that mutations resulting in extra or alternative disulfide bridges in the V1 and V2 domains may represent another mechanism by which HIV-1 generates structural diversity in order to evade the immune response.

Viruses with 17 cysteine residues, rather than the standard 18 cysteine residues, represent the largest single group of disulfide variants seen in this study (29/68 or 43%). When the location of the amino acid replacements resulting in the loss of a cysteine was examined, there was substantial clustering in the C1 and C2 domains, a pattern completely different from that observed for the double cysteine insertions described above (Fig. 1). Thus, 24 of the 29 cysteine replacements were located in the C1 and C2 domains or at regional boundaries near C1 or C2. Moreover, these cysteine replacements occurred exclusively in the N-terminal half of the molecule, remote from the V3 to C4 domains known to be essential for CD4 and chemokine receptor binding. The remaining five 17-cysteine mutants were found in V3, C3, V4, and C4 and their impact on receptor binding is under investigation.

Fifteen viruses were observed that contain an additional cysteine residue (i.e., 19) relative to the expected number of cysteine residues. As was noted for the molecules with 17 cysteines, there was clustering in the C1 and C2 domains in 7/15 viruses. Furthermore, 10/15 variants were located in the N-terminal portion of the molecule upstream of the V3 loop (as defined in Fig. 1A). The substitutions in viruses with 17 and 19 cysteines were localized to discrete regions of gp120, and these locations differed from the variants containing 20 cysteines. While the occurrence of the mutations did not reach statistical significance (see above), this clustering does suggest that the substitutions may have a discrete function that provides a selective advantage. To analyze the functionality of envelope proteins with unusual disulfide structures, pilot studies were carried out with three viruses containing 19 cysteine residues. For this purpose we employed the PhenoSense infectivity assay described by Richman *et al.*[13] Genes encoding three full-length gp160 envelope proteins were cloned into an expression vector and used to prepare stocks of pseudotype viruses. The resulting stocks were then tested for infectivity on U87 cell lines expressing CD4 and either the CCR5 or CXCR4 chemokine receptor (Table 2). The infectivity of the pseudoviruses with 19

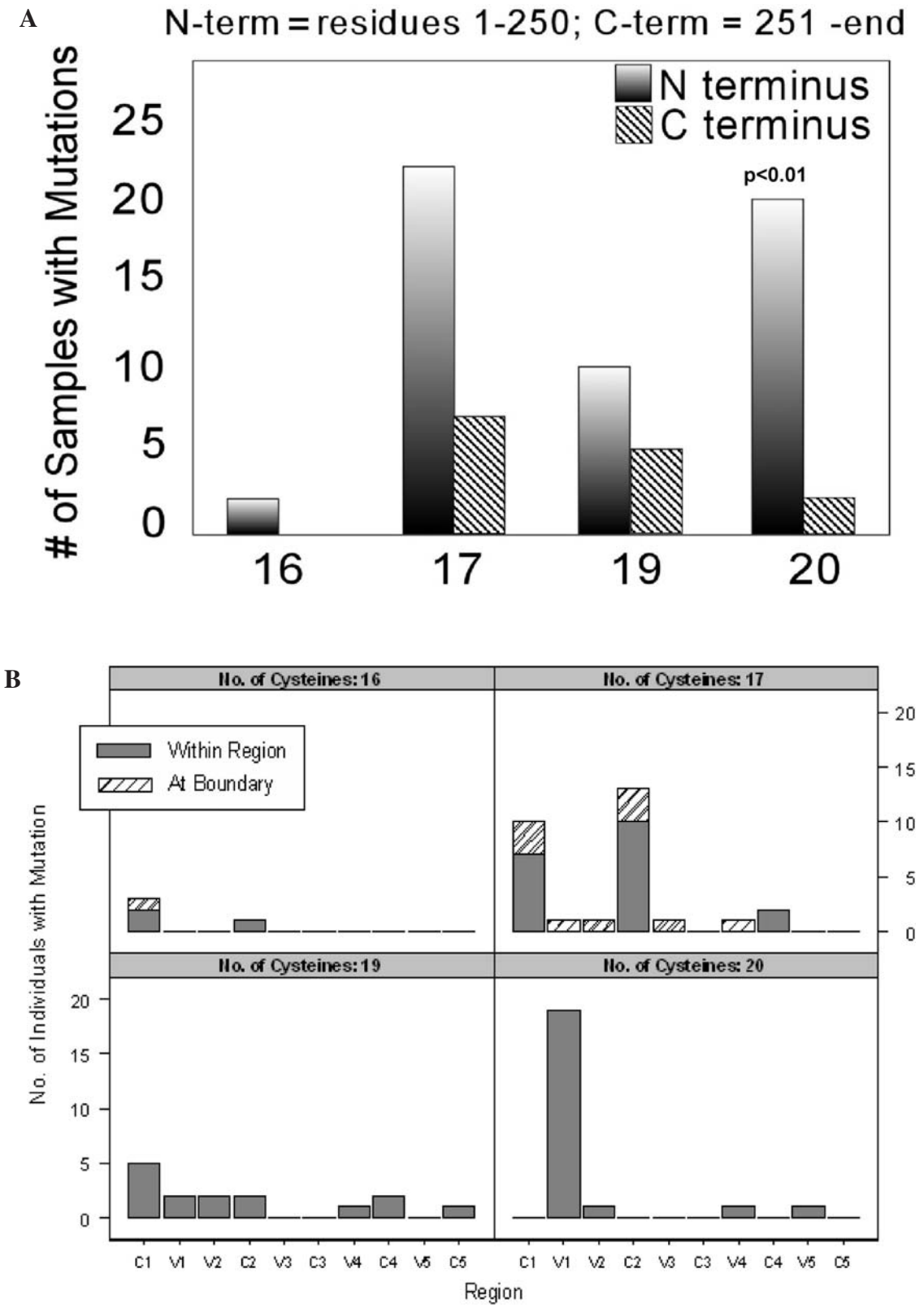

FIG. 1. Location of disulfide variants broken down broadly by N- and C-terminal regions (**A**) or by individual domains (**B**). Boundary mutations are those that occur at cysteine residues that define a variable or a conserved region (e.g., at C1/V1). Regions of clustering that are significant are indicated in (A). Statistical comparisons were made using Fisher's exact test for binomial proportions.

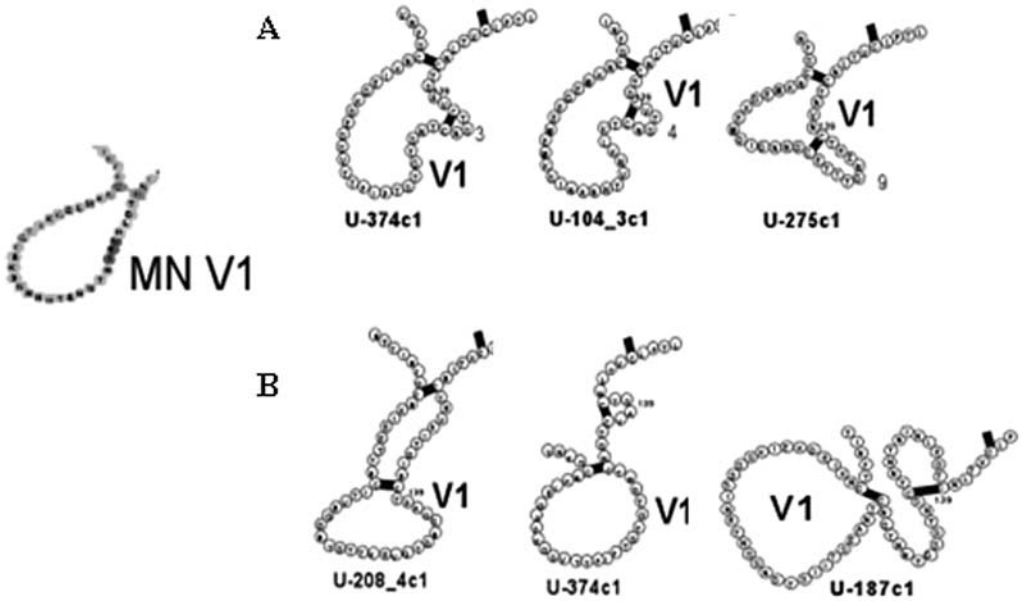

FIG. 2. Possible disulfide structures of mutants containing two additional cysteine residues in V1. For comparison, the conventional wild-type structure is depicted here by the MN strain. Diagrams in (A) suggest a simple loop formation is added to the wild-type loop creating an "oven mitt" structure. Numbers near the "thumb" region indicate the number of amino acid residues incorporated into the new loop. In (B), theoretical alternate structures (a "lariat" structure) are proposed that may substantially alter the V1 loop presentation. Designations shown under each loop (e.g., U-374c1) represent a code corresponding to a gp120 sequence derived from an individual clinical trial participant.

cysteines in gp120 was compared to that of standard control pseudoviruses that were CCR5 tropic, CXCR4 tropic, or dual tropic. When the results were analyzed we found that all three pseudoviruses tested were infectious and all three utilized the CCR5 chemokine receptor.

DISCUSSION

In this paper we describe polymorphism in the cysteine content of HIV envelope proteins from newly acquired subtype B infections. The type of variation we observed can be divided into two major types: (1) variation that adds or deletes two cysteine residues that potentially results in either 8 or 10 disulfide bridges, or (2) variation that deletes or adds one cysteine residue resulting in an unpaired cysteine residue and 8 or 9 disulfide bridges. To our knowledge, there has been only one previous report of an individual possessing a subtype B virus with an extra pair of cysteines in the V1 domain of gp120.[11,12] However, the fact that we have found 19 individuals with extra pairs of cysteines in the V1 domain suggests that cysteine content mutations in V1 are fairly common and may be of functional sig-

TABLE 2. INFECTIVITY PHENOTYPE OF ENVELOPE PROTEINS WITH 19 CYSTEINES[a]

Virus	*Approximate gp120 AA*[b]	*gp120 cysteines*	*gp120 glycosylation*	*R5 cell growth (RLUs)*[c]	*X4 cell growth (RLUs)*	*Tropism*
U-099c31	475	19	25	290,350	2,697	R5
U-183c27[d]	486	19	26	206,228	4,076	R5
U-209c23[d]	481	19	27	602,864	3,634	R5
JRCSF	474	18	23	>1,000,000	4,536	R5
92HT594	474	18	27	>1,000,000	>1,000,000	R5/X4
HXB2	481	18	24	406	360,016	X4
MN[e]	484	18	22	—	—	X4

[a]The infectivity assay was run according to Richman *et al.*[13]
[b]The gp120 amino acid length does not include the signal peptide sequence.
[c]RLUs, relative light units.
[d]Note that U-183 and U-209 have a cysteine mutation that occurs at the same position in V2.
[e]MN is included for comparison purposes only and was not used in the infectivity assay.

nificance. That this mutation has not been reported more frequently may relate to the fact that relatively few newly transmitted viruses have been sequenced, or that many of the gp120 sequences in the literature lack data corresponding to the C1 to V2 fragment of the envelope gene where many of the newly described cysteine mutations occur.

In addition to the V1 loop mutations, we found one individual each with two extra cysteines in the V2, V4, and V5 domains of gp120. Viruses with two extra cysteines in the V4 domain are a common feature of many naturally occurring subtype A/E strains[10] and are thought to be stable. Interestingly, we have completed an approximately 2-year longitudinal analysis of one dually infected trial participant whose major subtype B virus population contains mainly 20 cysteines in the V4 region and found the virus present over the entire interval (D.V. Jobes *et al*., unpublished data).

We found 44 individuals with envelope proteins containing an unpaired cysteine (15 with 19 cysteines and 29 with 17 cysteines). Viruses with 17 cysteines can form a maximum of 8 disulfide bonds compared to the normal 9 disulfide bonds. Interestingly these mutations result in viruses that lack covalent C1, C2, C4, V1, and V2 loop structures. This type of variation could potentially affect the antigenic structure of the molecule, however, further studies are required to confirm that these mutations all result in functional viruses. Free sulfhydryl groups, resulting from an uneven number of cysteines, are unusual in extracellular regions of proteins. One possible explanation for the presence of an unpaired cysteine residue in gp120 would be that it becomes paired with a complementary unpaired cysteine located in gp41. Covalent linkages between the receptor binding domain (e.g., gp120 homologue) and the fusion domain (gp41 homologue) are known for other retroviral envelope proteins (Pinter *et al*.[22] and references therein). However, preliminary examination of gp41 ectodomain sequences from 37 viruses with an uneven number of cysteines in gp120 failed to show the same number of significant unpaired cysteines. In fact, only 11 unique mutations occurred, resulting in the addition of an extra cysteine in gp41: three mutations from a glycine to cysteine and the remaining eight from a tyrosine to a cysteine (D.V. Jobes *et al*., unpublished data). In practice though, it is possible that these additional gp41 cysteine residues could mediate homodimer formation between adjacent gp41 molecules and do not in fact interact directly with gp120, but this scenario remains to be investigated. Another possibility is that the free cysteines mediate the formation of homodimers between two gp120s. While studies are ongoing to explore the likelihood of this structure, it is possible that such an arrangement could affect the trimeric nature of gp120/gp41[23–25] and impact infectivity by stabilizing a conformationally altered dimer that might displace the remaining unpaired gp120 (i.e., subunit shedding).

Another possibility is that the unpaired cysteines are able to form a mixed disulfide with another non-gp120 protein of cellular or viral origin to promote virus infectivity. Support for this alternative is suggested by studies reporting that redox changes in the D2 domain of CD4 can influence the binding of gp120 and hence virus entry, and that changes in the redox potential might be mediated by thioredoxin secreted by $CD4^+$ T cells.[26] In addition, Barbouche *et al*.[27] have noted a high density clustering of protein disulfide isomerase (PDI) on lymphocytes and have suggested that PDI may alter the conformation of HIV-1 envelope proteins by reducing cysteine bonds. A corollary of these PDI or thioredoxin-induced redox changes is that unpaired cysteines may provide a mechanism to generate viral diversity by allowing for the generation of alternative disulfide structures (discussed below).

Prior to this study there had been no direct evidence that viruses containing 17 or 19 cysteines were functional. Tschachler *et al*.[28] deleted cysteine residues from the V1, V2, V3, V4, and C4 domains of gp120 via *in vitro* mutagenesis and found that none of the progeny virus was infectious, with the exception of a mutation in the V4 domain, which was weakly infectious. However, these investigators did not consider mutations in the C1 and C2 domains where many of the unpaired cysteine variants observed in our studies were located. McCutchan *et al*.[29] also observed several individuals with amino acid replacements at conserved cysteines, but they were not tested for infectivity so it is not clear whether they are actually defective genomes. However, several observations that support the possibility that the 17- and 19-cysteine viruses may be common and functional include (1) *in vitro* assays of virus variants with 19 cysteines show robust infectivity values (Table 2); (2) the unpaired cysteines are not randomly distributed and tend to cluster in the C1 and C2 domains; (3) similar mutations have been observed in different individuals; and (4) the 17- and 19-cysteine variants are epidemiologically unlinked (based on phylogenetic analysis; unpublished data) and have been collected from infections occurring in different parts of the country.

Since few gp120 sequences from early infections have been described, it is possible that viruses with unusual disulfide structures represent a "transmission phenotype." Indeed Wang *et al*.[11] noted that viruses with 20 cysteines were observed early in infection and disappeared at later time points, although our own longitudinal study showed a 20-cysteine variant in V4 that persisted and dominated the virus population for the full 2 years of follow-up. Fortunately, sequential longitudinal specimens extending 2 years after infection are available for most of the viruses that we have described and will be used to document the relative stability of viruses with unusual cysteine content. Although thousands of gp120 sequences have been assembled in various sequence databases (GenBank, Los Alamos National Laboratories), most of these are from fragments of gp120 and most were collected from viruses long after the time of infection. It is well established that enormous virus variation evolves within individuals during the course of HIV-1 infection[30–32] and that the sequence of viruses that mediates infection may be different from the sequences that mediate disease. To date the vast majority of published HIV sequences were collected from subjects long after the time of initial infection. It may well be that the lack of success of previous vaccine development efforts may in part be due to the fact that vaccine antigens have been selected from prevalent virus infections rather than incident virus infections. In this regard the disulfide variation that we report in this paper, along with reports of short V region loops and fewer glycosylation sites in viruses from new infections,[6,33] may define characteristics of a transmission virus "phenotype." If so vaccines that contained antigens with these structural features would be expected to be more effective than vaccines containing antigens from viruses collected during the persistent or symptomatic phases of HIV infection. The data presented in this paper suggest that variation in cysteine content and disulfide structure is more common than previously appreciated and may represent a mechanism of immune escape employed

by HIV. A mechanism of antigenic variation resulting from disulfide bond shuffling could yield considerable variation in the structure of gp120 and may provide a means to escape immune attack. Currently, experiments aimed at understanding the functional significance of disulfide structure variation are in progress. These include studies of antigenic structure and neutralization with monoclonal and polyclonal antibodies and studies measuring the infectivity and viral fitness of envelope proteins from early infections with fewer or greater than 18 cysteine residues. In the future we also hope to carry out bioanalytical studies aimed at determining the actual structures of disulfide loops present in viruses with 16, 17, 19, and 20 cysteine residues.

Finally, it is apparent from analysis of full-length gp120 sequences collected from the AIDSVAX B/E Phase 3 vaccine trial conducted in Thailand (subtypes AE and B') that this pattern of unusual cysteine residues also exists (D.V. Jobes *et al*., unpublished data). It is possible then that a nonstandard gp120 cysteine content could be common for the HIV envelope protein and occur across other HIV subtypes like A, C, and D found in Africa. Therefore additional studies are warranted to determine if these other HIV subtypes also exhibit an unusual cysteine content.

SEQUENCE DATA

The GenBank accession numbers for the individual sequences are 1–68.

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