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Diversity in Virus Populations from Genital Secretions and Peripheral Blood from Women Recently Infected with Human Immunodeficiency Virus Type 1

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In order to develop a human immunodeficiency virus type 1 vaccine with global efficacy, it is important to evaluate the virus populations that are transmitted to individuals living in high-incidence areas. To determine the nature of the human immunodeficiency virus type 1 population transmitted to women during heterosexual contact, we examined the diversity of the proviral envelope gene in infected cells in both genital secretions and peripheral blood from six recently seroconverted Kenyan women. Heterogeneous virus populations were present in cervical secretions and/or peripheral blood shortly after seroconversion for five of six infected individuals, and tissue-specific variants were identified in several cases.

The human immunodeficiency virus type 1 (HIV-1) population in an infected person often includes a diverse mix of variants distinguished primarily by differences in the extracellular envelope glycoprotein. Because the envelope contains critical determinants of cell tropism (2, 3, 5, 6, 8, 11, 13, 18, 19, 24, 26), some variants may be favored for cell- and tissue-specific replication. This is well documented for chronic HIV-1 infections, in which variant virus populations that are distinct from those in peripheral blood exist in some tissues, including genital mucosa (1, 4, 7, 9, 14, 20, 22). However, analyses of virus populations in acute HIV-1 infection have focused exclusively on virus in peripheral blood. In contrast to the diversity found in virus from blood of chronically infected persons, virus from acutely infected individuals is reported to be homogeneous (17, 28–30). Because HIV-1 is transmitted by sexual contact, virus from blood of a newly infected person might not reflect the full spectrum of transmitted variants. Therefore, infecting strains of sexually transmitted HIV-1 might best be represented by provirus found in genital mucosa. In order to develop vaccine therapies designed to prevent new infections, it is important to identify and characterize the features of transmitted viruses.

In this study, we examined the envelope diversity and tissue specificity of heterosexually transmitted HIV-1 in a cohort of six female sex workers near the time of seroconversion. The women were participants in a PAVE (Preparation for AIDS Vaccine Evaluation) study in Mombasa, Kenya, a cohort study of HIV-1 seroincidence in which subjects were screened at approximately monthly intervals for sexually transmitted diseases (STDs) and HIV-1 antibodies (15). Positive enzyme-linked immunosorbent assay (ELISA) results were confirmed by immunoblot analysis. Subjects Q03, Q47, Q52, and Q84 had attended the clinic monthly for at least 3 months and had at least three negative HIV serologies prior to seroconverting (Table 1). Q45 seroconverted 1 month after her first clinic visit. Q23 had a negative test on her first clinic visit and was seropositive when she next visited the clinic 4 months later. The STDs that were detected at the clinic visits in the 90 days prior to the visit at which seroconversion was detected or at the seroconversion visit (the visit at 142 days post-negative serology was included for Q23) are summarized in Table 1. None of the women in this cohort had a history of blood transfusions or intravenous drug use. There was no evidence of epithelial disruption in the genital tract, and none of the women were menstruating at the time of sample collection.

Approximately 1 week following a positive HIV-1 test, peripheral blood mononuclear cells (PBMCs) and cervical swabs were collected. Cells from these samples were lysed, and aliquots were used directly for nested PCR amplification of a 1.2-kb fragment encompassing the coding region of the extracellular glycoprotein as described previously (20). Briefly, 0.1 to 5 μl of cells at a concentration of 10^4 cells per μl was used as a template for PBMC samples. All women had at least 1 infected cell per 10^4 PBMCs as determined by endpoint dilution. For cervical swabs, 5 μl of sample was used for all PCRs.

TABLE 1. Summary of the cohort and samples analyzed

Subject no.	Days PNS[a]	STDs[b]	No. of CX clones[c,d]	No. of PB clones[d,e]
Q84	60	0	18 (6)	15 (4)
Q03	57	0	10 (4)	10 (6)
Q52	35	GC, CT	5 (2)	17 (7)
Q45	49	0	13 (3)	12 (4)
Q47	67	TV, GUD	15 (6)	14 (5)
Q23	142	0	14 (5)	15 (6)

[a] Days PNS (post-negative serology) refers to the number of days between the last negative ELISA result and the time of sample collection.
[b] STDs diagnosed within the 90 days (142 days for Q23) prior to a positive HIV-1 ELISA result or at the time of the seroconversion visit. 0, no STD detected; GC, *Neisseria gonorrhoea*; CT, *Chlamydia trachomatis*; TV, *Trichomonas vaginalis*; GUD, genital ulcer disease.
[c] CX indicates a sample from a cervical swab.
[d] The number of clones analyzed for each tissue is listed, with the number of PCRs from which clones were isolated given in parentheses.
[e] PB indicates a PBMC sample.

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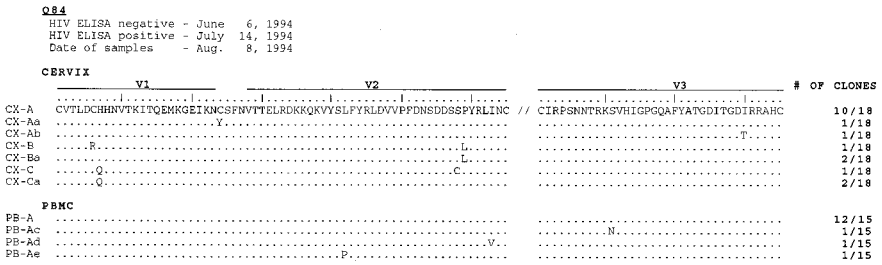

FIG. 1. Alignment of predicted amino acid sequences of V1, V2, and V3 of the HIV envelope gene from cervical secretions and PBMCs of Q84. Dates of sample collection, seroconversion, and last HIV-1 negative serology are given. A reference clone was chosen that most closely approximated a consensus sequence of all cervical clones. The entire sequence of this representative clone is given in single-letter amino acid code. The locations of V1, V2, and V3 loop are indicated above the sequence. Predicted amino acid differences from the reference sequence are shown for each clone, and similarities are indicated by dots. Deletions are indicated by vertical lines. Clones are named for the tissue sample (CX, cervix; PB, PBMC), and individual variants are designated by capital letters. Clones that differed by only one amino acid are considered related genotypes and are indicated by small letters (e.g., CX-Aa is considered a subclone of CX-A). The proportion of clones with a given sequence is indicated on the right of the sequence.

To increase the likelihood of detecting different proviral genomes, products from a minimum of four PCRs (representing a range of cell equivalents in the case of PBMC samples) were used for cloning (23). With two cervical samples, this was not possible because of low levels of detection of HIV-1 provirus (Table 1). The primer pair HIV*env*12 (CCTGGTGGGTGCT ACTCCTA) and HIV*env*13 (CCACTCTATTTTGTGCATC AGA) was used in the first round of amplification, and the pair HIV*env*9 (*CTACCCGGG*CATATGATACAGAGGTACATA ATGTCTGGGC) and HIV*env*10 (*CGCTCTAGA*CACTTCT CCAATTGTCCCTCAT) was used in the second round. The italicized residues introduced restriction sites for *Sma*I and *Xba*I into the amplified product to facilitate cloning. Fragments were purified and cloned into M13mp19.

We chose to evaluate proviral DNA, rather than viral RNA, because the goal of this study was to identify the virus population that was originally transmitted to these women. Recent reports indicate that the turnover time of viral RNA in plasma of chronically infected individuals is on the order of several days (10, 25), whereas provirus persists for the lifetime of the cell and may represent a better archive of earlier events. Viral RNA present at seroconversion, therefore, may represent viral selection actually in process in the host but not those genomes that were initially transmitted. We analyzed the sequences of three variable regions (V1, V2, and V3) in the extracellular glycoprotein for each clone because these regions affect cell tropism and antiviral immunity (2, 3, 5, 6, 8, 11–13, 16, 18, 19, 24, 26, 27, 31). Three patterns emerged from a comparison of proviral envelope gene sequences from each of these six subjects. These patterns were characterized by (i) homogeneity

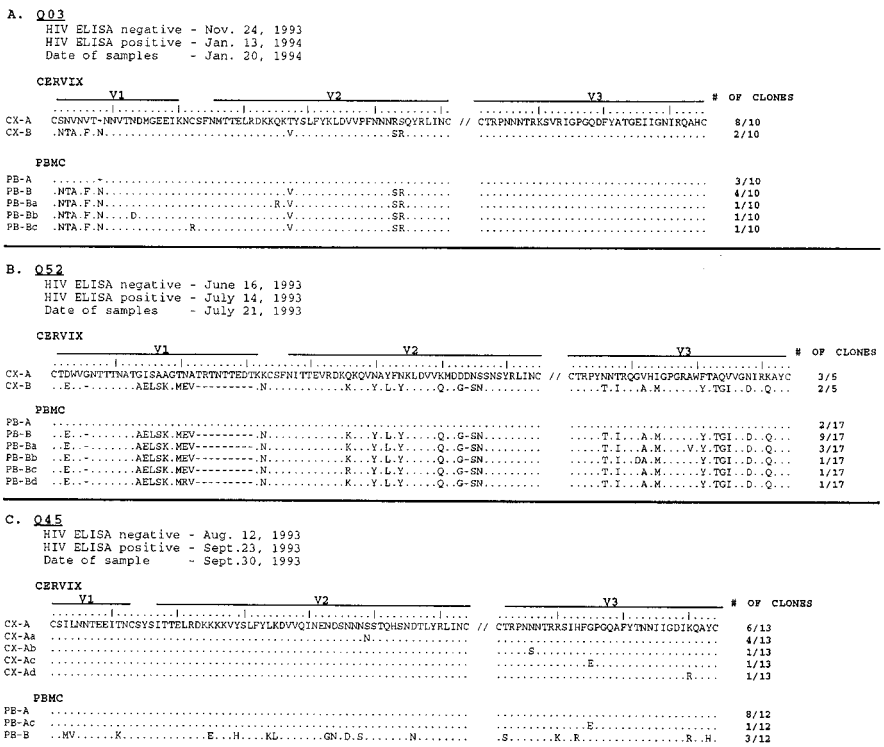

FIG. 2. Alignment of predicted amino acid sequences of V1, V2, and V3 of the HIV envelope gene from cervical secretions and PBMCs from Q03 (A), Q52 (B), and Q45 (C). The nomenclature and layout of this figure are similar to those described in the legend to Fig. 1.

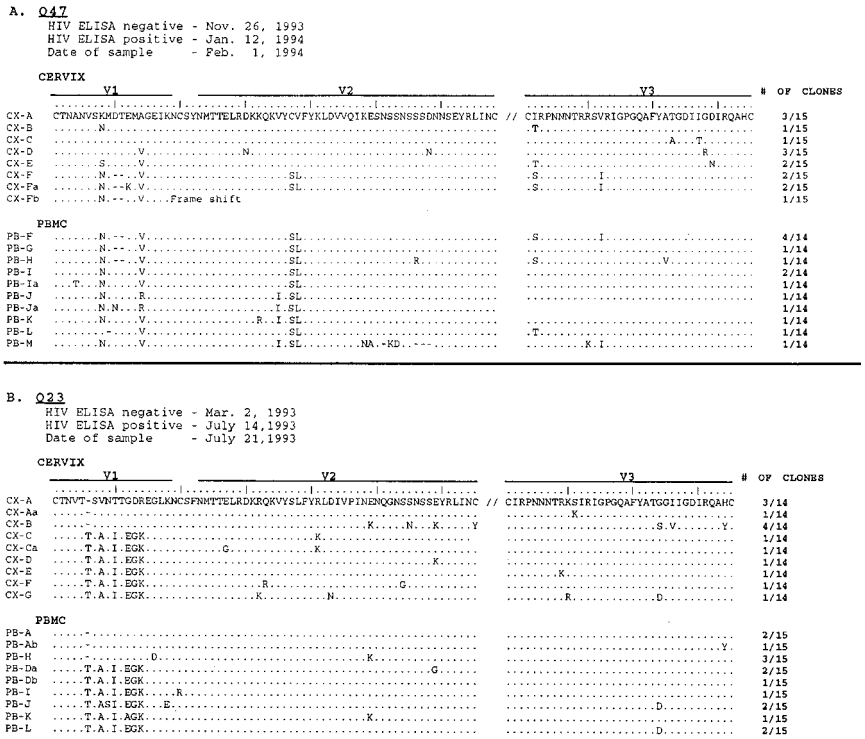

FIG. 3. Alignment of predicted amino acid sequences of V1, V2, and V3 of the HIV envelope gene from cervical secretions and PBMCs from Q47 (A) and Q23 (B). The nomenclature and layout of this figure are similar to those described in the legend to Fig. 1.

both within and between samples from cervical secretions and PBMCs, (ii) two distinct viral genotypes in either PBMCs or both cervical secretions and PBMCs with very little heterogeneity within each genotype, and (iii) multiple variants identified for each site.

Proviral sequences from subject Q84's cervical secretions and PBMCs were essentially homogeneous, with two minor variants detected in cervical secretions that differed in V1 and V2 from the major genotype by two amino acids. These minor variants were not found in clones from PBMCs (Fig. 1). None of the individual amino acid changes found in clones from cervical secretions were found in sequences from PBMCs.

In contrast to the viral homogeneity in Q84, two distinct viral genotypes were detected in three individuals from this cohort (Fig. 2). In clones from each of these individuals, the degree of heterogeneity differed among the variable regions, with V1 being the most divergent. Differences between variable regions for each pair of genotypes were determined by using the longest contiguous stretch of amino acids as the denominator, and insertions of any length were counted as one difference. For example, for subject Q03 (Fig. 2A) two variants (variants A and B) differed from each other in V1 (25%) and in V2 (8.3%) but were homologous in V3. There was very little heterogeneity within each genotype. Both variant A and variant B were detected in cervical secretions and PBMCs, but the distribution of genotypes was different between sites. Variant A represented 80% of the cervical clones (e.g., CX-A) but only 30% of the clones from PBMCs (e.g., PB-A), and this distributional difference was unlikely to be due to chance ($P = 0.07$). A similar pattern was seen in provirus populations from Q52 (Fig. 2B). In this case, the two genotypes differed in V1 (35.5%), V2 (23.7%), and V3 (29.4%). Again, both genotypes were found in cervical secretions and PBMCs, and the distribution of the two major genotypes varied between these sites. In Q52, CX-A represented 60% of all cervical clones while PB-A was found in only 12% of the clones from PBMCs ($P = 0.055$). Two variant genotypes that differed from each other in V1 (27.3%), V2 (18.8%), and V3 (14.7%) were also detected in Q45 (Fig. 2C). However, in this case only variant A was detected in cervical secretions.

A third pattern of viral diversity, characterized by multiple site-specific variants in both PBMCs and cervical secretions, was seen for Q47 and Q23 (Fig. 3). For Q47 (Fig. 3A), six distinct virus variants were found in cervical samples and the mean pairwise difference between all clones was 5.3%. Proviruses detected in cervical secretions differed from those detected in PBMCs (mean difference = 6.1%), and only variant F was common to clones from both sites. This pattern of multiple, diverse viral variants in both PBMCs and cervical secretions (mean difference = 5.6%) was also seen for Q23 (Fig. 3B), and only variant A was common to both sites.

This study demonstrates that there is virus heterogeneity in both cervical secretions and PBMCs in women soon after seroconversion. Diverse viral genotypes early in infection could result from divergence of a single transmitted variant, exposure to multiple strains of virus from different donors, or transmission of multiple genotypes during a single exposure. The extent of viral diversity within five individuals in our cohort and the short time of infection make it unlikely that variation was due to divergence of a single transmitted genotype. In support of this conclusion, very little envelope variation was detected in macaques inoculated with a simian immunodeficiency virus clone at 2 months after infection (21). Viral diversity in these individuals could also reflect multiple infections from different sexual encounters in a high-incidence area for HIV-1 infection. This cohort of sex workers is characterized by relatively low

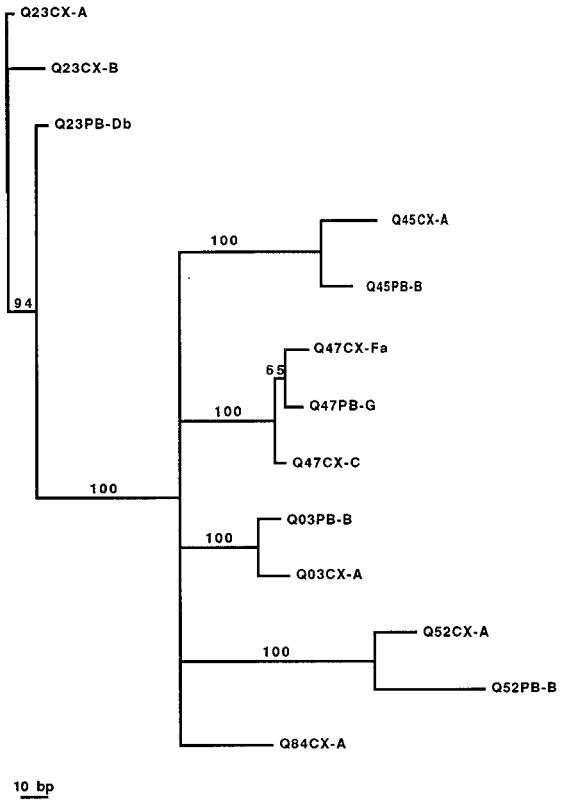

FIG. 4. Phylogram of representative viruses from all six individuals. The complete sequence of V1 through V3, including C2, was obtained and aligned by using the multiple sequence alignment function in the SeqApp program, and manual adjustments were made to improve alignment. The tree was the shortest tree generated by exact generalized unweighted parsimony by using PAUP 3.0s. Insertions and deletions do not affect distance in this program. Tree length was 487. Numbers at the nodes indicate the percent bootstrap values derived from 100 bootstrap resamplings. The scale at the bottom shows the branch length, representing 10 bp, and horizontal branch lengths are drawn to scale.

sexual frequency, with only two sexual encounters per week on average (15). Therefore, it is unlikely that each of the women would have been infected by multiple sex partners during a short period of time. However, if multiple infections had occurred, we would expect that intrapatient viral heterogeneity would be similar to interpatient viral diversity within this cohort. To examine this possibility, the phylogenetic relatedness of sequences encompassing V1 through V3 of representative clones from all six individuals was determined. Only one clone from Q84 was included in the phylogenetic tree because clones from this individual were essentially homogeneous. The tree generated was subjected to 100 random bootstrap resamplings, and results showed that clones from each individual clustered together (Fig. 4). Similar results were obtained when the analysis was based on the C2V3 region (data not shown). Thus, the observed virus diversity was unlikely to have resulted from exposure to virus from multiple donors.

Our results suggest that virus heterogeneity in this cohort was due to transmission of multiple HIV-1 genotypes. These observations contrast with previous studies showing a homogeneous virus population in PBMCs at or near seroconversion (17, 29, 30). Because these transmission studies each included analysis of different variable regions, direct comparison between cohorts is difficult. However, all investigators analyzed V3, and on the basis of V3 sequences alone, we would conclude that multiple variants were transmitted in four of the six cases that we evaluated. An important difference between our study and others is that the majority of individuals in previous cohorts were men (17, 29, 30). It is possible that transmission of heterogeneous virus populations is more common to women. However, transmission of a homogeneous virus population was reported by investigators who examined PBMC-derived virus from two women in the acute phase of HIV-1 infection (30). This apparent discrepancy in results was not due to nonsexual HIV-1 risk behaviors in our cohort, and there was no correlation in our study between infection with multiple HIV-1 genotypes and intercurrent STDs. Therefore, the difference in results between these studies may simply reflect the need for a larger sample size to detect transmission of diverse genotypes within a population. A final consideration is that pathogenesis of HIV-1 strains in Africa may differ from that of the more commonly studied B subtypes found in the United States and Europe. All women in our study were infected with viruses belonging to clade A, except for Q52, who was infected with clade D viruses (21a). Thus, our results underscore the need for studies of HIV-1 transmission and pathogenesis in geographically diverse settings.

This study is the first to compare proviruses derived from genital mucosa and PBMCs early in infection. We report that the population of viruses in genital secretions is not always mirrored by virus in PBMCs. For three individuals, two distinct genotypes that differed in their distribution between blood and genital mucosa were identified, and for two individuals of this cohort multiple genotypes unique to each site were detected. Our studies of chronically infected individuals also indicate that virus populations from mucosa and PBMCs may be distinct and that virus in genital mucosa may be most closely related to a minor subset of PBMC variants (20). These data may explain observations that virus in PBMCs of a person who has recently acquired HIV-1 infection through sexual contact may represent only a minor population of viruses in the PBMCs of the donor (30). Tissue-specific variant populations and distributional differences of variants in genital mucosa and PBMCs suggest that selective pressures on these two virus pools are different or nonsynchronous. This already-complex interaction of virus and host may be further complicated by effects of gender and viral subtypes. The mechanism by which HIV-1 subtypes are selectively transmitted and subsequently undergo clearance, sequestration, and evolution in different tissue compartments will be of paramount importance for understanding early immune responses against sexually transmitted HIV-1 and ultimately for designing globally effective vaccines.

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