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Evidence for Frequent Reinfection with Human Immunodeficiency Virus Type 1 of a Different Subtype†

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A major premise underlying current human immunodeficiency virus type 1 (HIV-1) vaccine approaches is that preexisting HIV-1-specific immunity will block or reduce infection. However, the recent identification of several cases of HIV-1 reinfection suggests that the specific immune response generated for chronic HIV-1 infection may not be adequate to protect against infection by a second HIV-1 strain. It has been unclear, though, whether these individuals are representative of the global epidemic or are rare cases. Here we show that in a population of high-risk women, HIV-1 reinfection occurs almost as commonly as first infections. The study was designed to detect cases of reinfection by HIV-1 of a different subtype and thus captured cases where there was considerable diversity between the first and second strain. In each case, the second virus emerged ~1 year after the first infection, and in two cases, it emerged when viral levels were high, suggesting that a well-established HIV-1 infection may provide little benefit in terms of immunizing against reinfection, at least by more-divergent HIV-1 variants. Our findings indicate an urgent need for studies of larger cohorts to determine the incidence and timing of both intersubtype and intrasubtype reinfection.

Human immunodeficiency virus type 1 (HIV-1) reinfection implies that a second viral strain can successfully establish infection despite prior infection with another HIV-1 strain. The first evidence that HIV-1 reinfection might be occurring came from cases where there was evidence for dual infection by viruses from different HIV-1 subtypes (2, 4, 6, 30). However, these cases were identified in cross-sectional studies, and it was therefore unclear when the different viruses were acquired: at the same time from a single coinfected source partner or sequentially from different partners. Nonetheless, both the frequent detection of cases of dual infection and the frequent detection of intersubtype recombinant viruses (22), which arise when a cell becomes infected by viruses from two distinct subtypes, imply that reinfection occurred in that individual or in someone in the transmission cascade.

Clear cases of HIV-1 reinfection, often called superinfection, are rare, perhaps in part because they can be detected only with careful longitudinal follow-up. The reported cases have been limited mainly to situations where treatment interruption has occurred or where reinfection occurred in the face of a virus that had low replication fitness and/or a different susceptibilities to antiretroviral drugs (1, 7, 9, 12, 13, 18, 24, 27, 31). These case reports indicated that both intersubtype reinfections, where viruses differ by ~30% in the viral envelope sequence, as well as intrasubtype reinfections, where viruses differ by only ~10% in the envelope, have occurred.

There have been a limited number of studies that examined the incidence of HIV-1 reinfections, and no cases were identified in the first such studies of U.S. and European populations (5, 8, 29). In these studies, the individuals were likely to have been exposed to a single subtype of HIV-1, subtype B, and the results suggested that the risk of intrasubtype B infections was lower than the risk of first infections in these cohorts. In a more recent study that included ~1 year of follow-up of 78 high-risk men who continued to be exposed to HIV-1 through sex with men, three cases of superinfection were observed (27). In each case, the reinfecting virus differed in its sensitivities to antiretroviral drugs from the initial strain, although it was unclear whether this was of significance in increasing the likelihood of the second infection. Interestingly, a high incidence of dual infection was observed within the first 3 months of infection in high-risk South African women, although reinfection was not detected over the next 1 to 2 years (10). These data suggest that in populations with a very high rate of partner exchange, reinfection may occur in the early phases after the first infection, perhaps implying that reinfections happen before HIV-1-specific immunity has fully developed.

To date, there have been no detailed longitudinal studies of the incidence and timing of HIV-1 reinfection in populations typical of the global HIV-1 epidemic, namely, individuals infected by heterosexual contact with antiretrovirus-naïve partners. To address this, we examined superinfection in high-risk Kenyan women who were enrolled in a prospective cohort and who were monitored from a point prior to HIV-1 infection through many years after acquisition (19). The present study shows that HIV-1 reinfection occurs quite commonly in women who continue to be exposed to HIV-1 through heterosexual contact and may occur well after the first infection is established.

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† Supplemental material for this article may be found at http://jvi.asm.org/.

MATERIALS AND METHODS

Study population. The prospective cohort of high-risk HIV-1–seronegative women from Mombasa, Kenya, from which our subjects were selected, has been

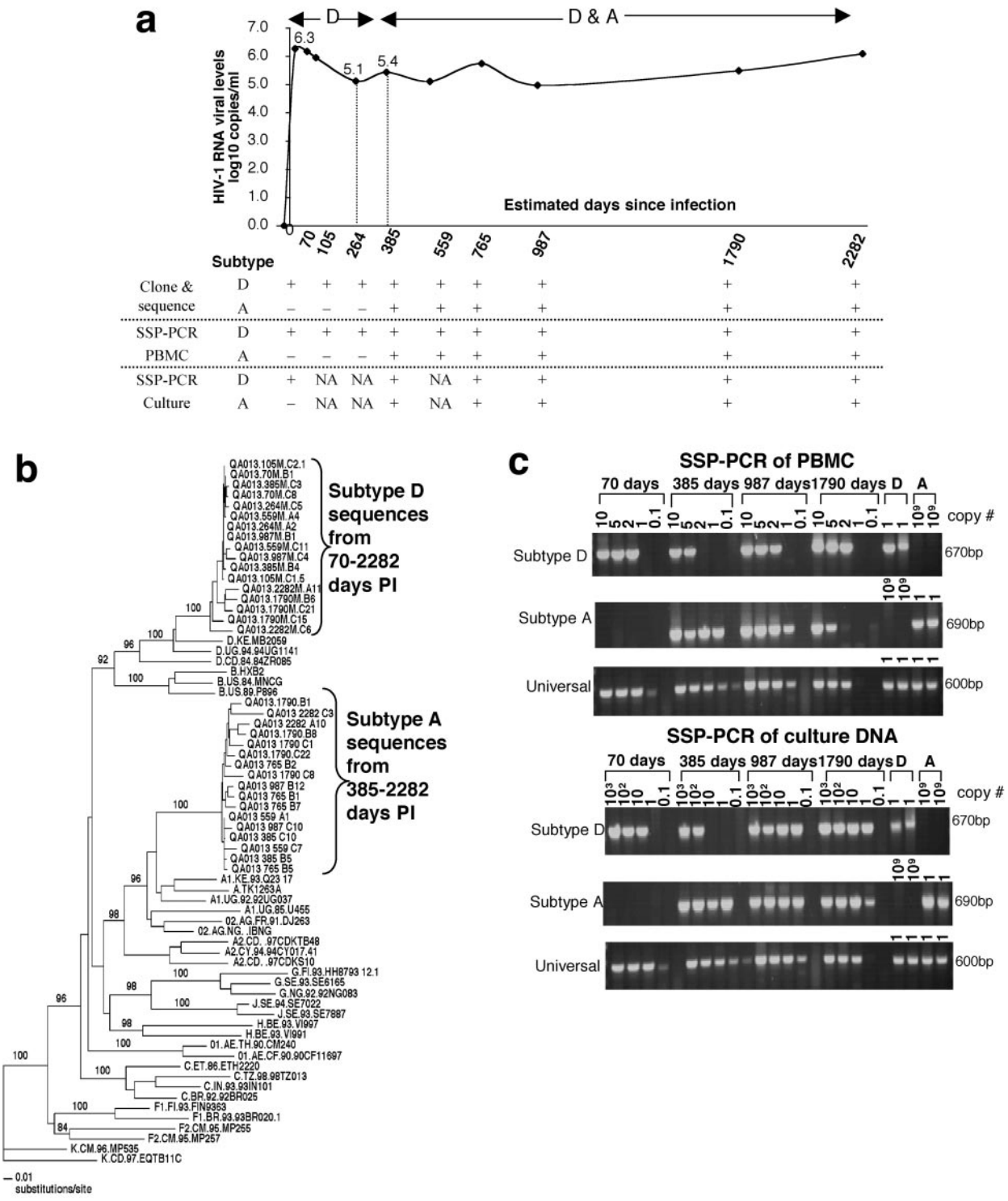

FIG. 1. Analyses of reinfection in subject QA013. (a) The graph illustrates HIV-1 plasma viral loads at various times postinfection. In this subject, both HIV-1 antibodies and RNA were detected 44 days after the subject had tested seronegative and HIV-1 RNA negative. Thus, the date of infection was estimated as the midpoint between the two visits, as described previously (14). Results of the three different methods of analysis of viral sequences are summarized below the indicated times p.i. The detection of subtype D or A sequences among clones obtained from the subject's PBMC DNA is shown on the top lines, with a + to indicate that at least one clone was present and with a − to indicate that no clones of that subtype were detected among those analyzed. Similarly, the presence or absence of a subtype-specific PCR product is shown with a + or −, respectively. NA means not available. The middle set of data is from analyses of the subject's PBMC DNA using SSP PCR, and the bottom set is from analyses of DNA from cells that were cultured to amplify virus. (Examples of these data are shown in panel c). (b) Phylogenetic analyses of sequences from different times p.i. by using a distance-based, neighbor-joining method. The numbers at the nodes represent bootstrap values for these nodes based on 100 bootstrap resamplings. Clones from the subject are designated QA013 followed by the days p.i. from which they were

described previously (19). Blood samples from these women were obtained every 1 to 3 months starting before HIV-1 infection and continuing thereafter. Infection was first detected by HIV-1 serology, and then the estimated date of infection was determined by testing stored retrospective plasma samples for HIV-1 RNA (14). None of the women reported using antiretroviral therapy at any time during follow-up. Informed consent was obtained from all participants. The study was approved by the ethical review committees of the University of Nairobi, the University of Washington, and the Fred Hutchinson Cancer Research Center.

Molecular analyses. The subtype of virus at the early time point was defined on the basis of envelope V1 to V3 sequences by using either the heteroduplex mobility assay or sequence analysis as described previously (17). For the samples from later time points, envelope fragments of 1.2 kb were amplified and cloned as described previously (17). For each sample, an average of 6 to 7 clones (range, 1 to 13 clones) was sequenced, and the subtypes of the V1 to V3 positions of the clones were determined in comparison to those of multiple reference sequences in the NCBI database (www.ncbi.nlm.nih.gov/retroviruses). Phylogenetic analysis was performed on the set of sequences obtained from different time points by aligning the sequences by using ClustalX with reference genomes from the Los Alamos database as well as other genomic sequences from this cohort. Aligned sequences were manually adjusted using MacClade (version 4.01), and the hypervariable regions with extensive insertions and deletions were masked using MacGDE (version 2.2). Phylogenetic trees were constructed using neighbor-joining trees based on Kimura's two-parametric distance estimates with the software package Phylogenetic Analysis Using Parsimony (and Other Methods) (28). Intersubtype recombination was determined by distance plotting and bootscanning using the sequences of subgenomic fragments with the Simplot program (16). The recombinant genomes were divided into distinct portions at the breakpoints (recombinant junctions), and phylograms were created for each section with the distance-based, neighbor-joining method by using PAUP.

Virus was amplified in culture from viably frozen peripheral blood mononuclear cells (PBMCs) by using standard methods. Briefly, 1×10^6 PBMCs were cultured with freshly phytohemagglutinin- and interleukin-2-stimulated PBMCs from HIV-1-negative donors. Cells were cultured for 21 to 28 days with the addition of 5×10^6 to 10×10^6 stimulated donor PBMCs at days 7 and 14. The DNA was extracted, and the HIV-1 proviral copy number was estimated by using real-time quantitative PCR with *pol*-specific primers as described previously (25). To be sure that the samples were from the same subject, human leukocyte antigen (HLA) typing was performed by using subtype-specific (SSP) PCR and a sequencing protocol for high-resolution typing of the HLA-A, HLA-B, HLA-C, and HLA-DPB1 genes (21).

SSP PCR. Primers were designed that were specific for the initial and reinfecting viruses for subjects QA013, QB008, and QB609. The primers were tested extensively for specificity and sensitivity by using envelope clones from each subject. In each case, the SSP primers could amplify a single copy of the desired template (except for primers to subject QB008, which could amplify ~10 copies; see Fig. 2). None of the SSP PCR primers amplified $\leq 10^9$ copies of the second virus in the subject.

DNA from PBMCs collected from the subject or from the viral culture was first amplified using a universal primer set that should detect all subtypes. This first-round PCR was then used as a template for second-round reactions with the subtype- and subject-specific primers or with an internal universal primer pair designed to amplify all subtypes. The primers and amplification conditions are as described in the supplemental material (Fig. S1).

Nucleotide sequence accession numbers. Representative viral envelope sequences from each time point for the three HIV-1 superinfection cases were submitted to GenBank as accession numbers DQ027773 to DQ027805.

RESULTS

For this study, we selected 21 women initially infected with subtypes C and D, which are less prevalent in Kenya, and looked for evidence of superinfection with A, the most prevalent subtype (20). The subtype of the virus, based on V1 to V3 envelope sequences at documented seroconversion, which was an average of 87 days postinfection (p.i.), was compared to the V1 to V3 subtypes 2 to 5 years later. For all but 4 of the 21 cases, the V1 to V3 subtypes of the initial and the later viruses were indistinguishable. In the four other cases, subtype A sequences were detected at the later time. In one of these, the HLA types of the samples were different, suggesting that the samples were not from the same subject, and this case was therefore excluded from further analysis. The remaining three cases were confirmed to be from the same subject by HLA typing, and HIV-1 sequences in longitudinal samples from these subjects (QA013, QB008, QB609) were further examined using a variety of methods.

Subject QA013. From subject QA013's PBMC DNA, only subtype D envelope clones were isolated at 70, 105, and 264 days p.i. (26 clones, 6 to 10 clones/time point). In contrast, envelope clones obtained from PBMCs collected 385 days p.i. and five time points thereafter were a mix of subtype D and subtype A at each time point (62 clones, 6 to 17 clones/time point) (Fig. 1a). All the subtype D sequences formed a monophyletic cluster and showed evidence of increasing diversification at a rate of ~1% per year, indicating that the initial subtype D virus continued to evolve throughout infection (Fig. 1b). Similarly, from 385 days p.i. onward, the subtype A sequences p.i. clustered together and diversified at about ~1%/year, further validating our suspicion that the subtype A virus had established a persistent infection.

We developed an SSP PCR method using primers designed to match the subject's early (subtype D) and late (subtype A) viral sequences to further examine the time of appearance of the second virus. These primers could detect a single copy of the desired template but did not amplify $\leq 10^9$ copies of the second virus (Fig. 1c). Only subtype D was detected by SSP PCR of PMBC DNA from 70 to 264 days p.i.; multiple attempts to amplify the subtype A envelope sequence from these early time points were negative (Fig. 1c and data not shown). In contrast, both subtype D and A products were detected in PBMC DNA at 385 days p.i. and all later time points. We also amplified the virus in this subject by culturing her PBMCs, and we then tested a higher number of HIV-1 genomes with SSP PCR (Fig. 1c). Subtype A could not be detected even when up to 10^3 HIV-1 copies, as quantified by real-time PCR, were sampled at 70 days p.i. Thus, HIV-1 subtype A was first detected by all three methods (PCR and cloning, SSP PCR of the subject's PBMC DNA, and SSP PCR of viral cultures) at 385 days p.i., but never before. This suggests that superinfection most likely occurred between 264 and 385 days p.i., a time

obtained and a clone designation such as A1 (where A indicates the PCR and 1 was the first clone from PCR A). The two clusters of different clones from this subject are marked with braces; one cluster groups with subtype D and one with subtype A, as indicated. (c) Results of subtype-specific PCR of PBMC DNA (top) and culture DNA (bottom) using subtype-specific inner primers. The samples are from the indicated days p.i. The amount of DNA that was added to the PCR mixture was based on the HIV-1 copy number in the sample, as indicated at the top of each lane. The last four lanes in each gel were PCRs of clones obtained from the subject that were known to be of one or the other subtype, as determined by phylogenetic analyses described above for panel b. Two different clones were tested at the indicated copy numbers, which were calculated from DNA concentration. In all cases, the product from the same first-round PCR was used for the indicated second-round PCRs; the primers for the second-round PCR are indicated to the right of the gels and are as described in the supplemental material (Fig. S1).

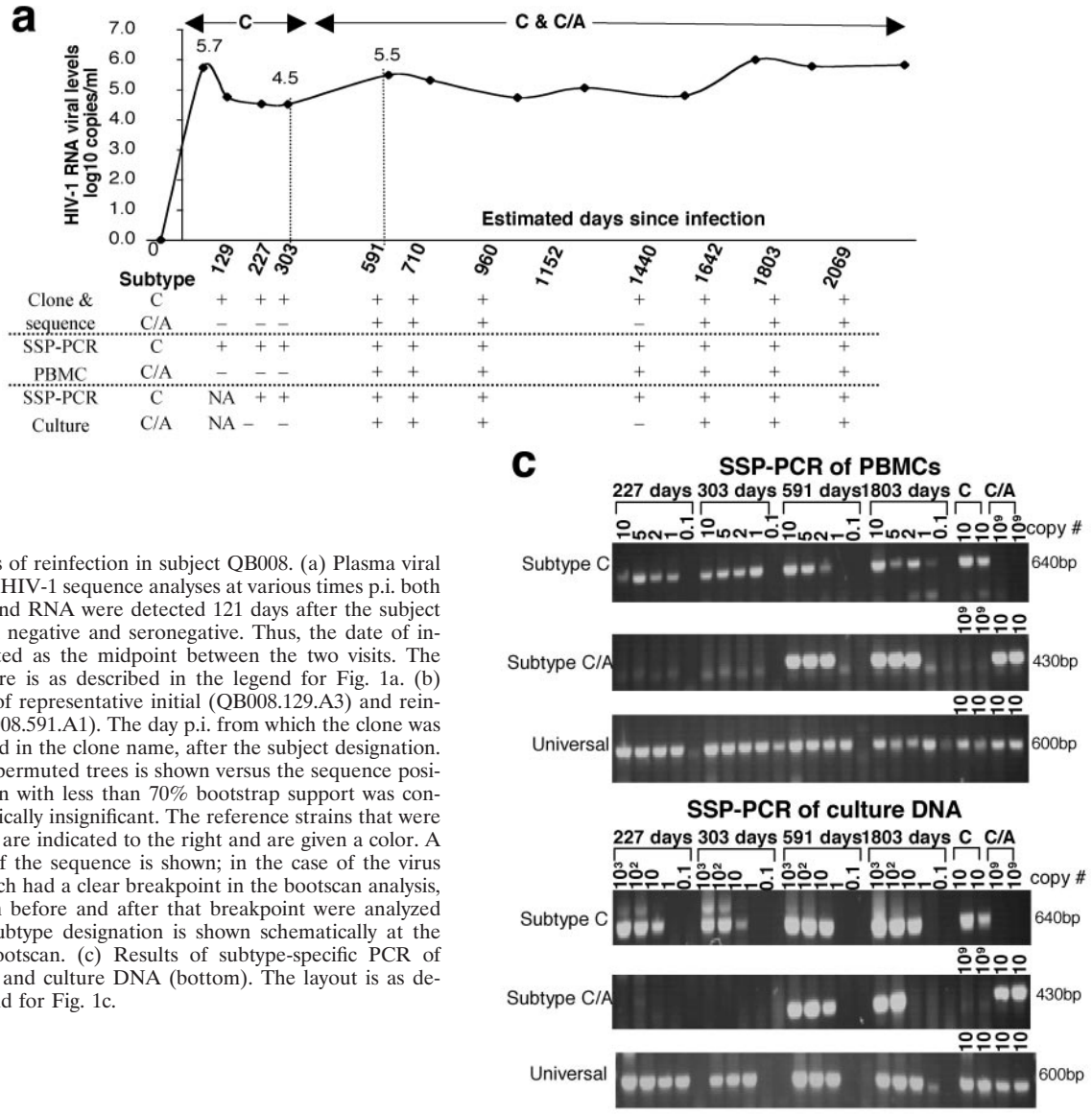

FIG. 2. Analyses of reinfection in subject QB008. (a) Plasma viral loads and results of HIV-1 sequence analyses at various times p.i. both HIV-1 antibodies and RNA were detected 121 days after the subject tested HIV-1 RNA negative and seronegative. Thus, the date of infection was estimated as the midpoint between the two visits. The layout for this figure is as described in the legend for Fig. 1a. (b) Bootscan analyses of representative initial (QB008.129.A3) and reinfecting clones (QB008.591.A1). The day p.i. from which the clone was obtained is indicated in the clone name, after the subject designation. The percentage of permuted trees is shown versus the sequence position. Recombination with less than 70% bootstrap support was considered to be statistically insignificant. The reference strains that were used in the analysis are indicated to the right and are given a color. A phylogenetic tree of the sequence is shown; in the case of the virus QB008.591.A1, which had a clear breakpoint in the bootscan analysis, the sequences from before and after that breakpoint were analyzed individually. The subtype designation is shown schematically at the bottom of each bootscan. (c) Results of subtype-specific PCR of PBMC DNA (top) and culture DNA (bottom). The layout is as described in the legend for Fig. 1c.

when plasma viral load was high ($>10^5$ $\log_{10}$ copies/ml) (Fig. 1a).

Subject QB008. In subject QB008, all of the envelope clones isolated at 129, 227, and 303 days p.i. were subtype C ($n = 26$). At 591 days p.i., there was a mix of subtype C and a new subtype C/A recombinant (10 and 4 clones, respectively) (Fig. 2a). Subtypes C and C/A were detected at all six time points examined thereafter ($n = 62$, 6 to 17 clones/time point) except 1,440 days p.i., when 10/10 envelope clones obtained from this subject were subtype C. The C/A recombinant carried subtype C sequences in V1 and V2, whereas the downstream sequences were more closely related to subtype A (Fig. 2b). The C/A recombinant sequences from all time points formed a monophyletic cluster (see Fig. S2 in the supplemental material) and continued to diversify (~1%/year), as did the subtype C sequences (~0.6%/year) (data not shown). Interestingly, the V1 to V2 portion of the C/A recombinant did not cluster more closely with the subtype C sequence detected earlier in this subject than with other subtype C viruses from this cohort or elsewhere (see Fig. S3 in the supplemental material), suggesting that the C/A recombinant was most likely not formed de novo in this subject.

SSP PCR gave results consistent with the analyses of V1 to V3 clones. Only subtype C was detected in PBMCs or viral-culture DNA from 129 to 303 days p.i. in QB008. In contrast, both C and C/A viruses were detected by SSP PCR at 591 days p.i., and seven time points thereafter, by using primers spanning the C/A recombination junction (Fig. 2c), as well as by using primers specific to the A portion of the sequence (data not shown). This suggests that superinfection with a C/A recombinant virus most likely occurred between 303 and 591 days p.i., a time when the viral load was ~10^5 $\log_{10}$ copies/ml.

Subject QB609. Subject QB609 also showed evidence of reinfection, although there was much more limited follow-up

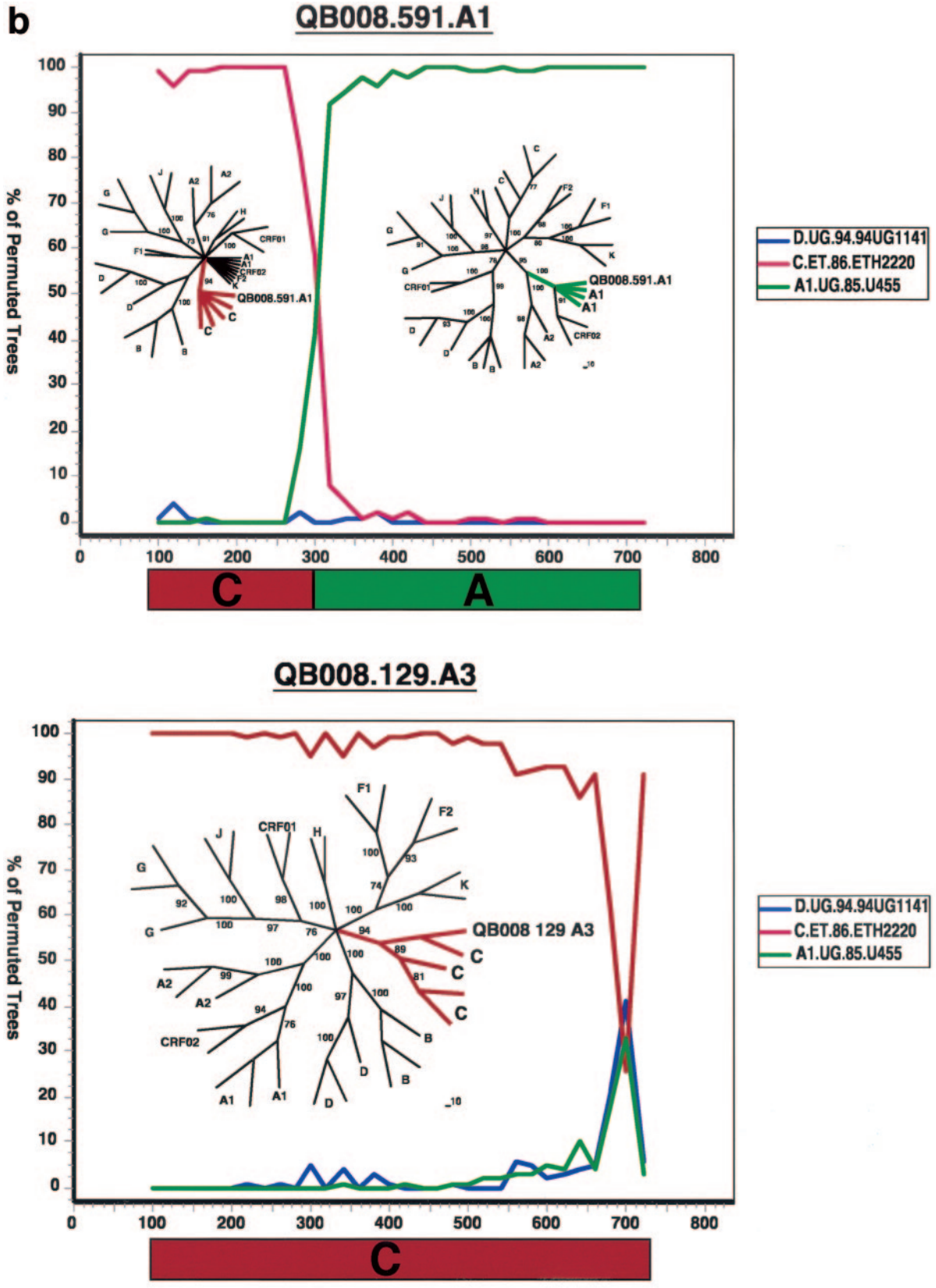

FIG. 2—*Continued*.

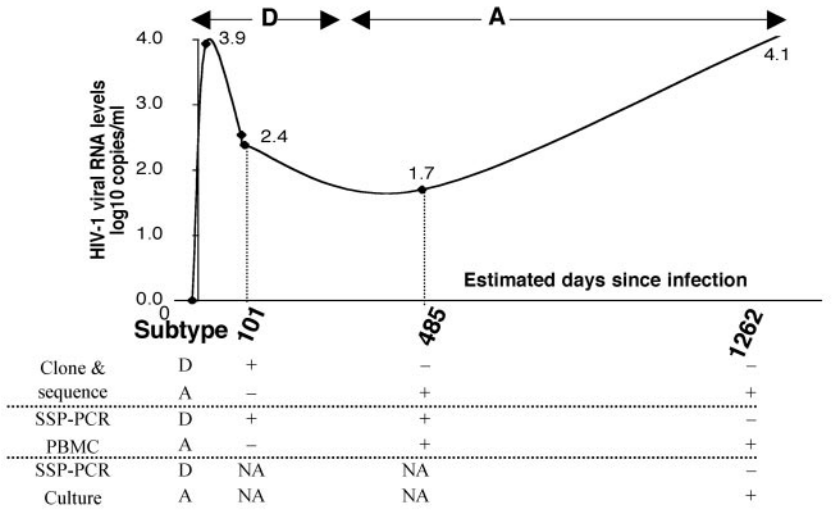

FIG. 3. Analyses of reinfection in subject QB609. This subject was found to be HIV-1 seropositive 77 days after testing seronegative. HIV-1 RNA was detected in plasma at the seronegative visit; thus, the time of infection was estimated as 17 days prior to that, as described previously (14). The layout is as described in the legend for Fig. 1a.

of this individual. HIV-1 envelope sequences from PBMCs collected 101 days p.i. were subtype D based on both cloning (six of six clones) and SSP PCR, whereas the sequences at 1,262 days p.i. were subtype A based on the results of both methods (four of four clones) (Fig. 3). One intervening sample was available at 485 days p.i., when the viral load was very low, and only three envelope sequences were obtained, all of which were subtype A. The subtype A sequences from 485 and 1,262 days p.i. clustered together in a phylogenetic analysis (see Fig. S3 in the supplemental material), confirming that the virus at 485 days p.i. was the same A variant found several years later. At 485 days p.i., both subtypes A and D were detected using SSP PCR. This suggests that superinfection with subtype A most likely occurred between 101 and 485 days p.i.

DISCUSSION

This study represents the first detailed longitudinal analyses of HIV-1 reinfection in populations at risk through heterosexual contact and suggests that HIV-1 reinfection may be quite common. The 20 women examined here are part of much larger cohort with over a decade of follow-up, during which the seroincidence was 10.3 cases per 100 person-years (for years 1993 to 2000 [L. Lavreys et al., unpublished data]). A total of 70 person-years of follow-up had accrued among the 20 women between the time when the first sample was detected at seroconversion and the time of the later sample, which would translate to seven cases of HIV-1 transmission if these women were HIV-1 naïve. Our study most likely underrepresented the cases of reinfection because in women where the second strain did not become dominant, the viral sequences may not have been represented among the limited number of clones examined. Moreover, we would have missed cases due to the limited numbers of clones sequenced and in instances where recombination led to a virus with the V1 to V3 sequences of the initial strain. Thus, these data suggest that the rate of incidence of reinfection is high and may approach the rate of incidence of initial infection.

The HIV-1 exposures in this group of women were relatively low compared to those of other so-called sex worker populations, because two-thirds of the women in this cohort are bar workers who commonly engage in sex work only to supplement income, not as a primary source of support (11, 19). Based on the reported sexual frequency (one to two partners per week) (15) the women would have had ~100 high-risk exposures in the time frame from initial infection to reinfection, which would correspond to a per-contact risk of reinfection of about 1%. This is similar to the risk of HIV-1 acquisition in HIV-1-naïve women (3), further supporting the suggestion that HIV-1 reinfections may be nearly as common as first infections.

In one of the cases identified here, QB609, the second infection occurred when the woman had a very low level of replication of the first virus. In this case, viral load was approximately 2 log units lower at both primary infection and near the presumed time of reinfection than is typical for African women (15). Thus, the initial virus may have been less fit, which is consistent with the observed later dominance of the superinfecting virus. However, in the other two cases, reinfection occurred in the face of a high level of virus replication, and the initial and reinfecting strains persisted at similar levels throughout infection. For example, in the case of subject QA013, reinfection occurred when plasma viral RNA was $>10^5$ copies/ml, and both the initial subtype D virus and the superinfecting subtype A virus could readily be detected throughout the ~5 years of follow-up after superinfection. This indicates that reinfection is not limited to cases where the initial viral strain is of low replication fitness.

In this study, we biased our study towards detecting intersubtype HIV-1 reinfection because we initially selected individuals infected with subtypes that are relatively rare in the population (23). By focusing on infections with a different subtype, which are expected to differ by ~30% in the envelope,

we could easily distinguish two such viruses from de novo variation, which is typically in the range of 1% per year (26). It is possible that more-related viruses would not be as successful at reinfection, although a recent study suggested that intrasubtype reinfection in high-risk men may also be relatively common (27). It will be important to assess the relative risk of intra- versus intersubtype reinfection with larger cohort studies, as this may provide important information when considering whether vaccine design should include region- or subtype-specific strains. At present, it is unknown whether susceptibility to reinfection is influenced by whether the viruses are of the same or different subtypes.

In all three cases identified in this study, the second virus was detected at the same time p.i. by multiple methods. The midpoint of the estimated window of reinfection was an average of 355 days p.i., a time when specific immune responses would be expected to develop and potentially broaden but well before clinically apparent immunodeficiency disease. While we could not confirm that the emergence of the second virus occurred on the heels of an exposure to such virus, this temporal linkage has been documented in a previous case report, where the second virus was detected a few weeks after a known exposure (12). In this case, reinfection with a second subtype also occurred after chronic infection was established but well before AIDS (~2.5 years p.i.) (12). Thus, these data suggest that the immune responses to the first virus provided limited benefit against infection by a second virus of a different subtype. These findings indicate an urgent need for larger studies of closely monitored cohorts to determine if there is a window when HIV-1 reinfection is more likely and whether this corresponds to a time before or after immune responses have broadened. These data suggest that vaccine strategies that are designed to mimic responses present in natural HIV-1 infections are unlikely to be fruitful. It will therefore be critical to define the responses that are lacking in individuals who become reinfected so that improving these responses can be the focus of vaccine development efforts.

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