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Founder Virus Population Related to Route of Virus Transmission: a Determinant of Intrahost Human Immunodeficiency Virus Type 1 Evolution?

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We and others have shown that in individual human immunodeficiency virus type 1 (HIV-1) infection, the adaptive evolution of HIV-1 is influenced by host immune competence. In this study, we tested the hypothesis that in addition to selective forces operating within the host, transmission bottlenecks have an impact on HIV-1 intrahost evolution. Therefore, we studied the intrahost evolution of the V3 region of the external glycoprotein gp120 of HIV-1 during the 3- and 5-year periods following seroconversion after parenteral versus sexual (male-to-male) transmission in 41 participants of the Amsterdam prospective cohorts of homosexual men (n = 31) and intravenous drug users (IVDUs; n = 10) who were AIDS free and had comparable numbers of $CD4^+$ cells. We observed that HIV-1 strains in homosexual men accumulated over 5 years more nonsynonymous substitutions within the V3 loop than HIV-1 strains in IVDUs as a result of lower rates of nonsynonymous evolution in both the initial 3-year period from seroconversion and the following 2-year period as well as a larger proportion of nonsynonymous back substitutions in IVDUs. The mean numbers of synonymous substitutions did not differ between the two risk groups. Since HIV-1 strains in IVDUs could be distinguished from the viruses of homosexual men based on several nucleotide substitutions of which the most conserved is a synonymous substitution at the tip of the V3 loop (GGC pattern), we studied whether the founder virus population itself has an impact on the intrahost evolution of HIV-1. The mean number of nonsynonymous substitutions accumulated over 5 years within the V3 loop was lower in 10 IVDUs infected by the HIV-1 strains with the GGC signature than in 4 IVDUs infected by HIV-1 strains lacking this pattern, while the mean numbers of synonymous substitutions were similar in the two groups.

Human immunodeficiency virus type 1 (HIV-1) causes a persistent infection in human hosts that ultimately results in AIDS. Extensive genetic variation of HIV-1 is usually observed within infected hosts in the course of individual HIV-1 infections (intrahost evolution). Previously we studied the intrahost evolution of HIV-1 during the initial 5-year period of infection following male-to-male sexual transmission (21). When the evolution of the consensus or master sequence coding for the V3 region of the HIV-1 envelope glycoprotein gp120 was studied in 44 infected individuals in relation to their clinical status or immune competence, no difference was seen in the mean numbers of synonymous substitutions between individuals who developed AIDS during the observation period (progressors) and AIDS-free individuals (nonprogressors). In contrast, the mean number of nonsynonymous substitutions accumulated over a 5-year period was significantly lower in progressors than in nonprogressors. The rate of nonsynonymous substitutions within a host correlated with the duration of the immunocompetent period as evaluated by the number of days until the $CD4^+$ cell number dropped below 200 per μl (21). This observation is in accord with results obtained by others (8, 37). Taken together, our data indicated that in the course of individual HIV-1 infections, virus populations continuously adapt to the intrahost environment and that this environment is shaped at least in part by antiviral immunity. More powerful and versatile intrahost environments of nonprogressors might require more adaptation than the defenseless environment of progressors. Our results suggest that host immune competence, as part of the intrahost environment, influences virus evolution.

Male-to-male transmission of HIV-1 represents a form of bottleneck transmission. It has been shown that the HIV-1 sequence population in a newly infected individual is relatively homogeneous and generally represents only a minor subpopulation of HIV-1 strains found in the donor (39). Virus or virus-producing cells have to be passed to the seminal fluid prior to virus transmission through homosexual intercourse. This process is associated with the restriction of HIV-1 quasispecies compared to that present in plasma or peripheral blood mononuclear cells (40). Later the virus is carried to the cells of the mucosa in the colorectal wall. Compartmentalization of HIV-1 in intestinal tract has also been shown (32, 33). Characterization of HIV-1 isolates recovered from bowel biopsies suggested that these strains may differ in biological properties from those recovered from serum of the same individual (2).

From this line of reasoning, it follows that in addition to intrahost selective forces, transmission bottlenecks may influence the rate of adaptation within the host. Some HIV-1 variants may be highly fit immediately following a transmission bottleneck, resulting in relative evolutionary stasis, as is seen in subsequent progressors. The rapid destruction of the immune system in such cases maintains a state in which the adaptive immune system has no selective power. Some other HIV-1 variants may be less fit immediately after transmission, which subsequently results in relatively rapid virus evolution, as is seen in nonprogressors. In part, this evolutionary process is accelerated by competent adaptive immunity that remains intact for a longer period of time.

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In this study, we attempted to test the hypothesis that parenteral transmission leads to faster intrahost adaptation (slower evolution) relative to sexual (male-to-male) transmission. Therefore, we obtained the sequences of the V3 region of the external glycoprotein gp120 of HIV-1 from 41 participants of the Amsterdam prospective cohorts of homosexual men (n = 31) and intravenous drug users (IVDUs; n = 10) at seroconversion as well as 3 and 5 years later and studied the intrahost HIV-1 evolution following parenteral versus sexual (male-to-male) transmission. Individuals from the two study groups did not differ in clinical status (AIDS free during the observation period) or laboratory markers of disease progression ($CD4^+$ cell numbers at seroconversion and at the 5-year time point, the mean $CD4^+$ cell decline over a 5-year period, HIV-1 RNA plasma loads at the 5-year point, and virus phenotype). This study was facilitated by the fact that the AIDS epidemic among IVDUs in Amsterdam is founded by HIV-1 strains that genetically differ from those present in homosexual men and can be distinguished based on one amino acid and two synonymous nucleotide substitutions in the sequence region studied, of which the most conserved is a synonymous nucleotide substitution in the second glycine codon at the tip of the V3 loop (GGC pattern) (16–18, 22). All viruses from IVDUs in this study group possessed this marker, while all viruses from homosexual men lacked it. In an attempt to distinguish the influence of the transmission route on HIV-1 intrahost evolution from that of the founding virus population, we studied HIV-1 evolution over a 5-year period in 14 additional seropositive IVDUs. Four of these viruses lacked the GGC marker and were closely related to viruses of homosexual men, as determined by the phylogenetic analysis.

MATERIALS AND METHODS

Study population. Serum samples were obtained at seroconversion as well as 3 and 5 years later from 41 participants of the Amsterdam prospective cohorts of homosexual men (n = 31), who seroconverted in 1985 to 1988, and IVDUs (n = 10), who seroconverted in 1986 to 1990. All individuals were AIDS free during the observation period. The mean $CD4^+$ cell numbers in those individuals were 676 ± 314 (mean ± standard deviation) and 600 ± 215 per μl at seroconversion and 426 ± 191 and 321 ± 134 per μl at the 5-year time point; the mean $CD4^+$ cell declines over 5 years were 250 ± 231 and 279 ± 223 per μl for homosexual men and IVDUs, respectively ($P > 0.1$ for all comparisons). The first HIV-1 isolates obtained from all individuals were shown to have the non-syncytium-inducing phenotype. During the observation period, a switch from non-syncytium-inducing to syncytium-inducing (SI) phenotype was observed in 5 of 31 homosexual men (16%) as well as in 2 of 10 IVDUs (20%; $P > 0.1$). The mean HIV-1 RNA copy numbers determined by the quantative NASBA technique (34) in serum samples obtained at the 5-year point were $10^{4.54 \pm 0.71}$ and $10^{4.55 \pm 0.56}$ for homosexual men and IVDUs, respectively ($P > 0.1$).

In addition, the V3 sequences were obtained from serum samples of 14 IVDUs for whom the moment of seroconversion is unknown since they entered the cohort HIV-1 seropositive. The sequences were derived from the first available sample and after 5 years of observation.

Sequence analysis. The procedures for viral isolation, reverse transcription, amplification, and sequencing were described earlier in detail (9). Briefly, RNA was isolated from 50 or 100 μl of serum by the method of Boom et al. (4). Virus RNA was transcribed into cDNA by using the 3′-V3-NOT primer. Part of each sample on which no reverse transcription step was performed and an extraction sample to which no serum was added were used as negative controls. The obtained cDNA was subjected to a nested PCR. The outer primers used for the first PCR were 5′-V3-NOT and 3′-V3-NOT; the inner primers used for the second PCR were SP6-5′-ksi and T7-3′-ksi. Nested PCR resulted in the amplification of a sequence approximately 270 bp in length. Double-stranded sequencing was performed on an automatic sequencer (model 373A; Applied Biosystems, Foster City, Calif.) with the *Taq* polymerase dye primer sequencing kit (Applied Biosystems). When direct sequences yielded illegible positions, nested PCR products were cloned by using the TA cloning system (Invitrogen, San Diego, Calif.), and a consensus sequence of six clones was used for analysis (in total, six samples had to be cloned).

Nucleotide sequences were aligned manually. All positions with an alignment gap in at least one sequence were excluded from any pairwise sequence comparison. Synonymous and nonsynonymous nucleotide p-distances (K_s and K_a) between the seroconversion, 3-year, and 5-year sequences from the same individual were calculated by using the MEGA program (20). The K_s/K_a ratio for the group of individuals was calculated according to the formula $K_s/K_a = (\Sigma M_{si}/\Sigma S_{si})/(\Sigma M_{ai}/\Sigma S_{ai})$, where ΣM_{si} and ΣM_{ai} represent the sum of intrahost (weighted) mutation events at coding synonymous and nonsynonymous sites, respectively, and ΣS_{si} and ΣS_{ai} represent the sum of intrahost (weighted) coding synonymous and nonsynonymous sites, respectively. The phylogenetic analysis was performed by using the PHYLIP package (neighbor-joining method) (12); the distance matrix was generated by using Kimura's two-parameter model (14). Multivariate principal coordinate analysis was done by using the PCOORD software (13). Signature pattern analysis was performed as described by Korber and Myers (15). Statistics were calculated by using the SPSS/PC+ software (version 5.0; SPSS Inc., Chicago, Ill.). The t test was used to compare groups.

RESULTS

In the first part of our study, we compared the intrahost evolution of the gp120 V3 region over a period of 3 and 5 years from seroconversion in 31 homosexual men and 10 IVDUs, who did not progress to AIDS during the follow-up period. Phylogenetic analysis of the sequence set revealed that viral evolution did not separate the sequences obtained at seroconversion from those obtained from the same individual 3 (data not shown) and 5 years later (Fig. 1), in accord with our previous observations for homosexual men (21). Also in agreement with our earlier findings (22), both the neighbor-joining phylogenetic and multivariate principal coordinate (PCOORD) methods separated the sequences obtained from homosexual men and IVDUs in two clusters according to risk group (Fig. 1 and data not shown). The signature pattern method revealed that one amino acid and two synonymous nucleotide positions differ significantly between the sequences obtained from IVDUs and homosexual men. The most conserved difference between the risk groups was a synonymous nucleotide substitution in the second glycine codon at the tip of the V3 loop (GGC) (16, 17, 19), which was seen in 10 of 10 sequences obtained from IVDUs but in none of 31 sequences obtained from homosexual men ($P < 0.0001$). The second distinction was found at position 15 of our sequences ($HIV\text{-}1_{LAI}$ *env* position 837), where 8 of 10 (80%) of IVDU sequences had a synonymous nucleotide substitution (TCC), compared to 1 of 31 (3%) of the sequences from homosexual men ($P < 0.001$). A risk group-associated amino acid signature pattern was seen at amino acid position 14 of our sequences ($HIV\text{-}1_{LAI}$ *env* codon 288), where 28 of 31 (90%) of the sequences from homosexual men had a Thr residue, compared to 2 of 10 (20%) of the sequences from IVDUs ($P < 0.001$).

The comparison of the seroconversion and 5-year sequences from each individual revealed that overall, in 5 years amino acid changes occurred at 26 of 35 positions of the V3 loop (105 nucleotides in length), which is the central functional part of the V3 region. The most variable were positions 308 and 320, where amino acid changes were observed in 19 of 41 individuals. The frequency of amino acid changes at these positions did not differ significantly between the risk groups. Changes at positions 308 and 320 occurred, respectively, in 5 and 4 (50 and 40%) of 10 IVDUs and 14 and 15 (45 and 48%) of 31 homosexual men ($P > 0.1$ for both comparisons). A positive amino acid residue at position 306, associated with the SI phenotype, was present in a 5-year sample of one homosexual man.

Comparison of the seroconversion and 5-year sequences from the same individual revealed a difference in the evolution of the V3 loop between the two study groups (Fig. 2). HIV-1 strains present in homosexual men accumulated over 5 years significantly more nonsynonymous substitutions than those present in IVDUs ($1.10 \cdot 10^{-2} \pm 0.54 \cdot 10^{-2}$ versus $0.60 \cdot 10^{-2} \pm 0.58 \cdot 10^{-2}$ per site per year; $P < 0.01$), while the mean numbers of synonymous substitutions did not differ between the two risk groups ($0.35 \cdot 10^{-2} \pm 0.44 \cdot 10^{-2}$ and $0.32 \cdot 10^{-2}$

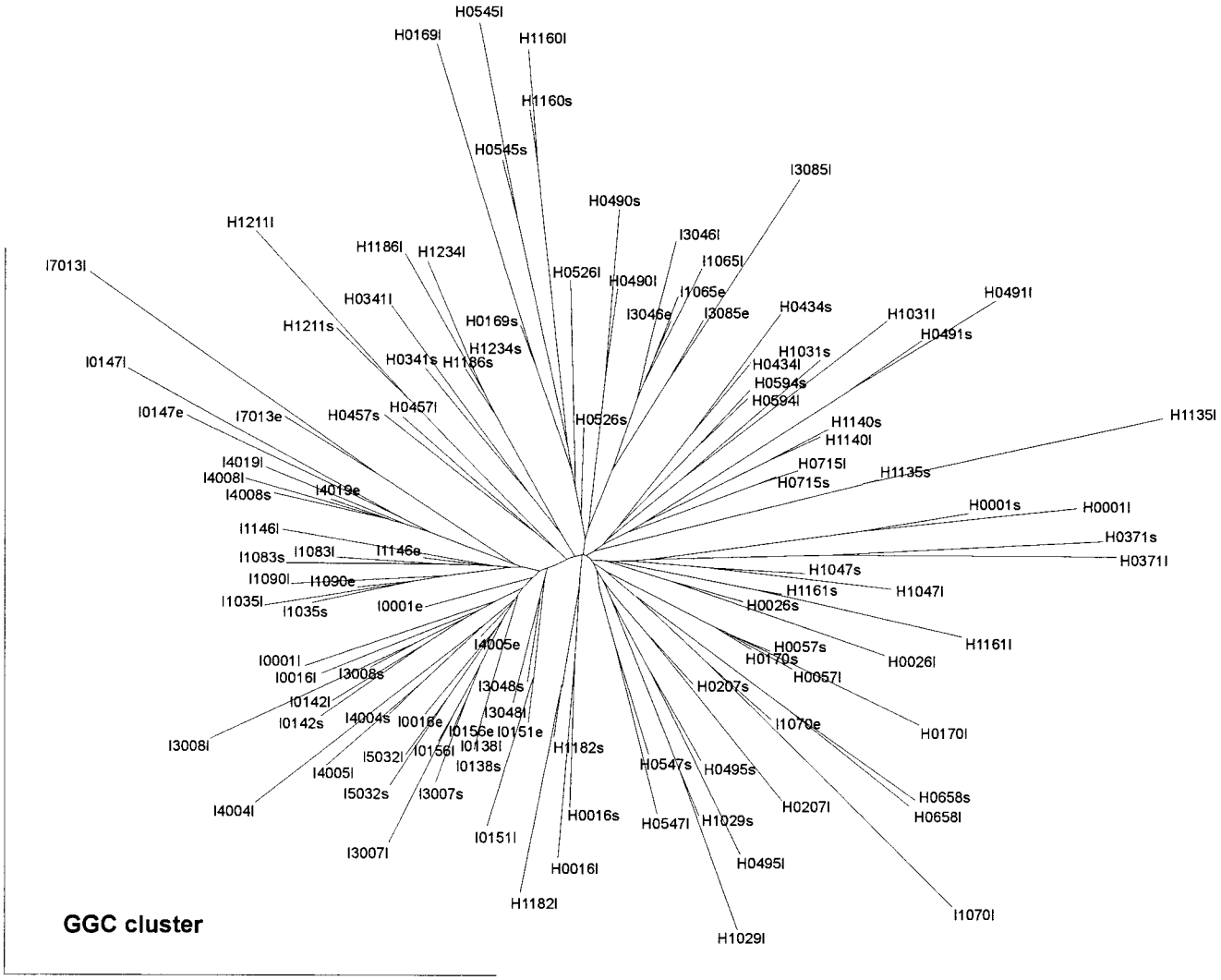

FIG. 1. Phylogenetic tree of the V3 sequences (270 nucleotides in length) derived at seroconversion (or from the first seropositive sample available) and 5 years later. Numbers indicate patient codes. H, homosexual men; I, IVDUs; s, seroconversion sequence; e, early sequence; l, late (5-year) sequence. The cluster of IVDU sequences with the GGC pattern is indicated.

$\pm 0.56 \cdot 10^{-2}$ per site per year, respectively; $P > 0.1$). The K_s/K_a ratio was higher in IVDUs than in homosexual men (0.55 versus 0.35). The mean amino acid distance between the seroconversion and 5-year sequences of the V3 loop was significantly higher in homosexual men than in IVDUs (0.12 ± 0.05 versus 0.06 ± 0.06; $P < 0.01$).

Subsequently we examined whether faster evolution of the V3 loop in homosexual men is a result of rapid adaptation immediately following HIV-1 transmission. To do so, we obtained the 3-year sequences from both experimental groups and analyzed the intrahost evolution of the V3 loop over the initial 3-year period and a subsequent 2-year period separately. The mean numbers of synonymous substitutions did not differ between the risk groups: $0.37 \cdot 10^{-2} \pm 0.60 \cdot 10^{-2}$ and $0.41 \cdot 10^{-2} \pm 0.66 \cdot 10^{-2}$ per site per year for the initial 3-year period, and $0.90 \cdot 10^{-2} \pm 1.51 \cdot 10^{-2}$ and $0.60 \cdot 10^{-2} \pm 0.97 \cdot 10^{-2}$ for the following 2-year period, for homosexual men and IVDUs, respectively ($P > 0.1$ for both time intervals) (Fig. 2). The mean numbers of nonsynonymous substitutions in both time intervals were higher in homosexual men: $1.16 \cdot 10^{-2} \pm 0.75 \cdot 10^{-2}$ and $0.79 \cdot 10^{-2} \pm 0.69 \cdot 10^{-2}$ per site per year for the initial 3-year period ($P > 0.1$), and $1.66 \cdot 10^{-2} \pm 1.15 \cdot 10^{-2}$ and $0.94 \cdot 10^{-2} \pm 0.68 \cdot 10^{-2}$ for the following 2-year period ($P = 0.02$), for homosexual men and IVDUs, respectively (Fig. 2). A markedly higher nonsynonymous evolution rate observed in both risk groups during last 2 years compared to the entire 5-year period was a result of multiple nonsynonymous back substitutions observed in this period, which were undetectable when the seroconversion sequences were compared to the 5-year sequences. We found that in IVDUs, 67% of nonsynonymous nucleotide substitutions that occurred between the 3- and 5-year time points were substitutions back to the seroconversion sequence, compared to 37% of nonsynonymous back substitutions in homosexual men ($P < 0.01$). When we compared the 3- and 5-year amino acid sequences, the mean number of amino acid changes over this period in homosexual men was 2.42 ± 1.67, of which 0.63 ± 0.88 (26%) were amino acid changes back to the seroconversion sequence. The mean proportion of back amino acid changes in IVDUs was significantly larger, 47% (0.78 ± 0.67 of 1.67 ± 1.00; $P < 0.01$). When sequences in which no amino acid changes occurred between the 3- and 5-year points were excluded from the analysis (two

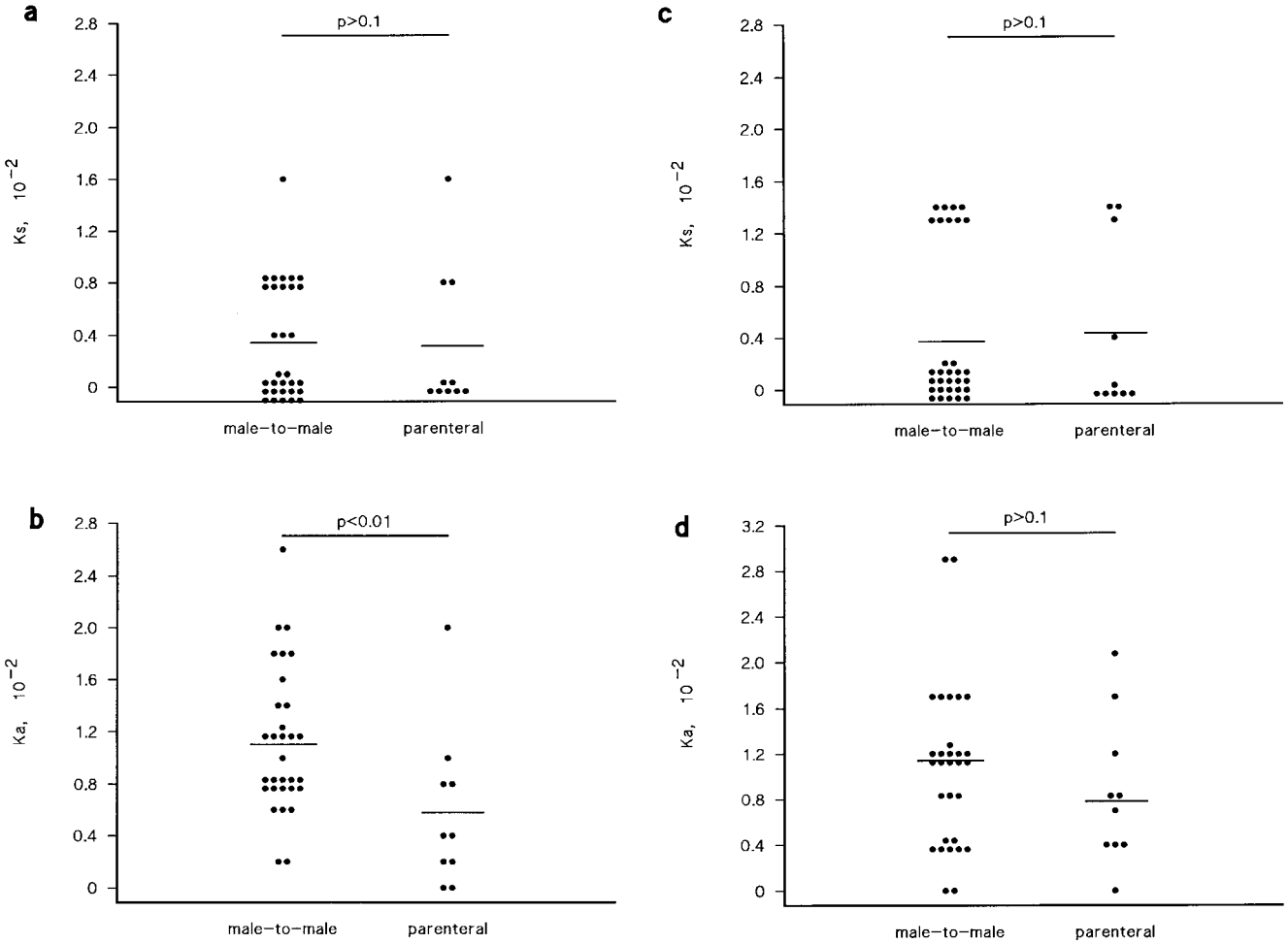

FIG. 2. Numbers of synonymous (a, c, and e) and nonsynonymous (b, d, and f) nucleotide substitutions per site per year observed within the V3 loops (105 nucleotides in length) of HIV-1 strains in homosexual men and IVDUs over the entire 5-year period of observations (a and b) as well as during the initial 3-year period (c and d) and the following 2-year period (e and f) separately. Horizontal lines indicate means.

from homosexual men and one from an IVDU), the proportions of back amino acid changes were 21 and 60% for homosexual men and IVDUs, respectively ($P < 0.005$).

To rule out the possibility that the difference in the intrahost evolution of the V3 loop shown in Fig. 2 could be due to slightly different mean $CD4^+$ cell numbers in these two groups of individuals, we analyzed whether these parameters are associated. We observed no significant association between the numbers of synonymous or nonsynonymous substitutions accumulated over a 5-year period and the $CD4^+$ cell numbers either at seroconversion or after 5 years or with the $CD4^+$ cell decline over a 5-year period ($P > 0.5$ for all regression analyses [Fig. 3]). Moreover, a significantly faster intrahost evolution of the V3 loop in homosexual men was still observed when the groups were adjusted for $CD4^+$ cell numbers at seroconversion (three homosexual men with relatively high $CD4^+$ cell numbers at seroconversion [>1,000 cells/ml] shown in Fig. 3 were excluded from the analysis [data not shown]).

When the flanking regions of the V3 loop (sequence 270 nucleotides in length) were also included in the analyses, the mean numbers of nucleotide substitutions in the V3 region were $0.93 \cdot 10^{-2} \pm 0.34 \cdot 10^{-2}$ per site per year for homosexual men and $0.78 \cdot 10^{-2} \pm 0.30 \cdot 10^{-2}$ per site per year for IVDUs ($P > 0.1$). Again the mean number of nonsynonymous substitutions per nonsynonymous site in the whole V3 region was higher in homosexual men, but this difference lost its significance ($1.03 \cdot 10^{-2} \pm 0.40 \cdot 10^{-2}$ and $0.88 \cdot 10^{-2} \pm 0.38 \cdot 10^{-2}$ per year for homosexual men and IVDUs, respectively; $P > 0.1$). In both groups, the mean numbers of nonsynonymous substitutions per nonsynonymous site per year were significantly higher ($P < 0.001$ for both cases) than the mean numbers of synonymous substitutions per synonymous site per year ($0.37 \cdot 10^{-2} \pm 0.38 \cdot 10^{-2}$ and $0.32 \cdot 10^{-2} \pm 0.36 \cdot 10^{-2}$ for homosexual men and IVDUs, respectively).

Because of a genetic difference between HIV-1 strains circulating in both risk groups that we had noticed previously (16–18, 22), we subsequently addressed the issue of whether this in itself influences the intrahost evolution. Since all AIDS-free IVDUs in the Amsterdam cohort from whom the seroconversion and 5-year samples were available were infected by the IVDU HIV-1 strains (with the GGC pattern in the second glycine codon at the tip of the gp120 V3 loop), in the hope of recovering non-GGC viruses among IVDUs, we analyzed HIV-1 sequences present in 14 IVDUs for whom no seroconversion date is known since they were positive upon entry into the cohort. The sequences were derived from the first available HIV-1-positive sample as well as after 5 years. The V3 sequences obtained from 10 of 14 individuals contributed to the

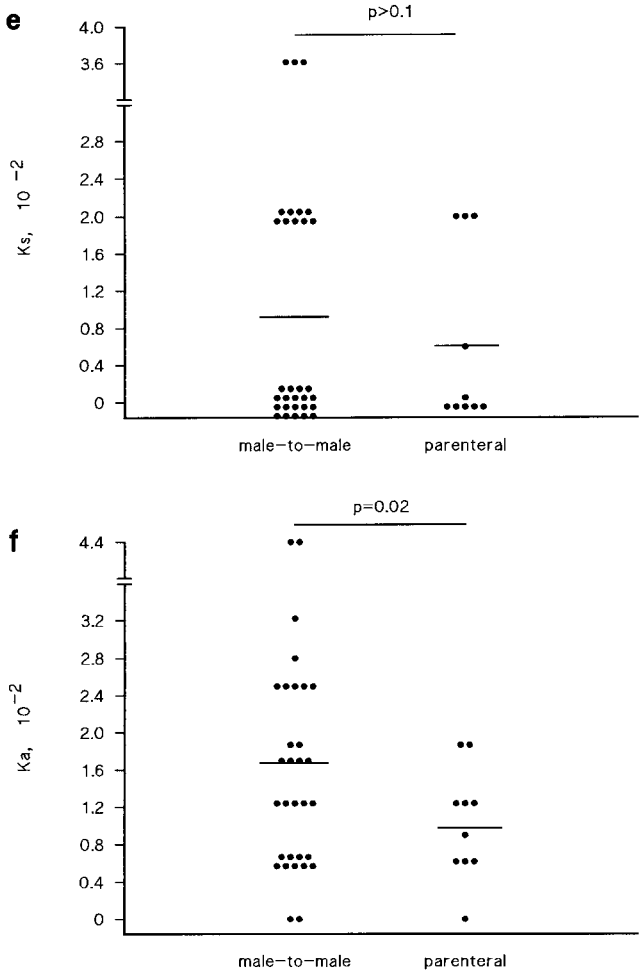

FIG. 2—*Continued.*

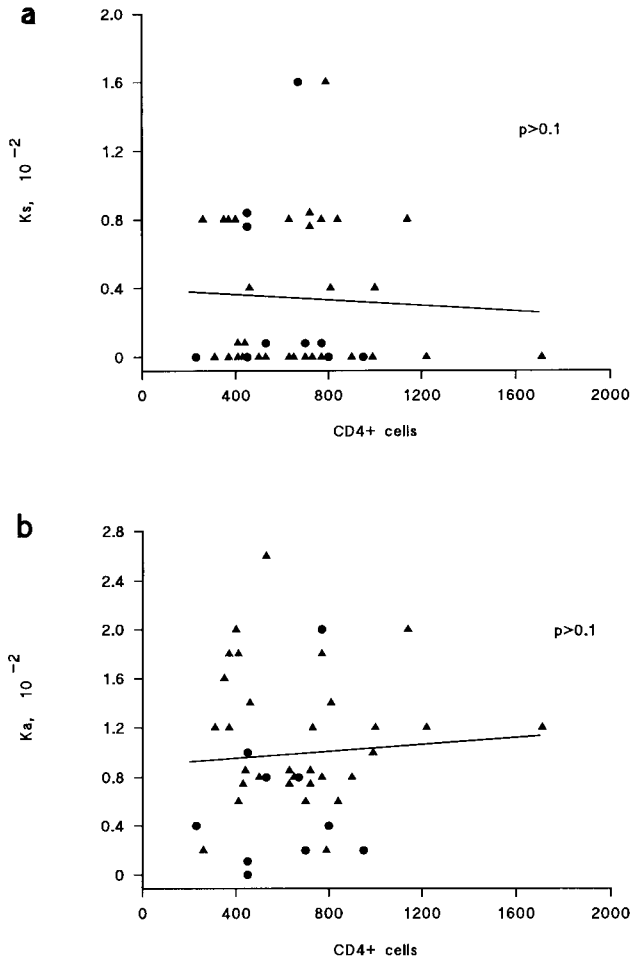

FIG. 3. Numbers of synonymous (a) and nonsynonymous (b) nucleotide substitutions per site per year within the V3 loop (vertical axes) in relation to the $CD4^+$ cell numbers at seroconversion (horizontal axes). Circles, homosexual men; triangles, IVDUs. (a) $r = -0.052$, $r^2 = 0.003$, $P = 0.75$; (b) $r = 0.067$, $r^2 = 0.004$, $P = 0.68$. Similar analyses for the $CD4^+$ cell numbers at the 5-year time point and for the $CD4^+$ cell decline over a 5-year period also revealed no significant association ($P > 0.5$ for all analyses).

IVDU phylogenetic cluster (Fig. 1) and carried the GGC and TCC markers, which were conserved during the observation period. The V3 sequences from another four individuals (without the GGC and TCC pattern) had Thr residues at position 14 and clustered together with the V3 sequences from homosexual men, as determined by the phylogenetic and PCOORD analyses (Fig. 1 and data not shown).

The mean number of nonsynonymous substitutions per nonsynonymous site per year within the V3 loop was significantly lower in individuals infected by the HIV-1 strains with the GGC pattern than in those infected by HIV-1 strains without this pattern ($0.73 \cdot 10^{-2} \pm 0.52 \cdot 10^{-2}$ versus $1.32 \cdot 10^{-2} \pm 0.20 \cdot 10^{-2}$; $P < 0.01$ [Fig. 4]). The mean numbers of synonymous substitutions per synonymous site per year within the V3 loop were similar in the two groups ($0.39 \cdot 10^{-2} \pm 0.55 \cdot 10^{-2}$ and $0.23 \cdot 10^{-2} \pm 0.49 \cdot 10^{-2}$, respectively; $P > 0.1$).

DISCUSSION

Homosexual men and IVDUs form the two main risk groups for HIV-1 infection in most of the European and North American countries. It has been shown that the natural histories of HIV-1 infection in these groups differ in several aspects, including the immunological and clinical status of the individuals, the clinical markers for AIDS progression, the relative prevalence of clinical symptoms of AIDS, the spectrum of the opportunistic infections (26–28, 30), prevalence and incidence of the SI viruses (31), etc. However, no investigations have been performed so far to compare the intrahost evolution of HIV-1 strains in homosexual men and IVDUs.

In this study, as in our earlier study (21), we used the rate of accumulation of nucleotide substitutions per site per year as a measure of the evolution rate. The study focused on the evolution rate of the consensus or master sequence obtained by the direct sequencing of a total pool of HIV-1 genome variants present in plasma.

Previously we and others have shown that the rate of intrahost evolution of HIV-1 is strongly influenced by the intrahost environment (8, 21, 36, 37). The rate of accumulation of nonsynonymous substitutions that reflects virus adaptation under selection pressure was shown to be significantly higher in immunocompetent asymptomatic individuals than in individuals who developed immunodeficiency and progressed to AIDS and was strongly associated with the length of the immunocompetent period (21). In contrast, the rates of accumulation of synonymous substitutions were similar in progressors and nonprogressors.

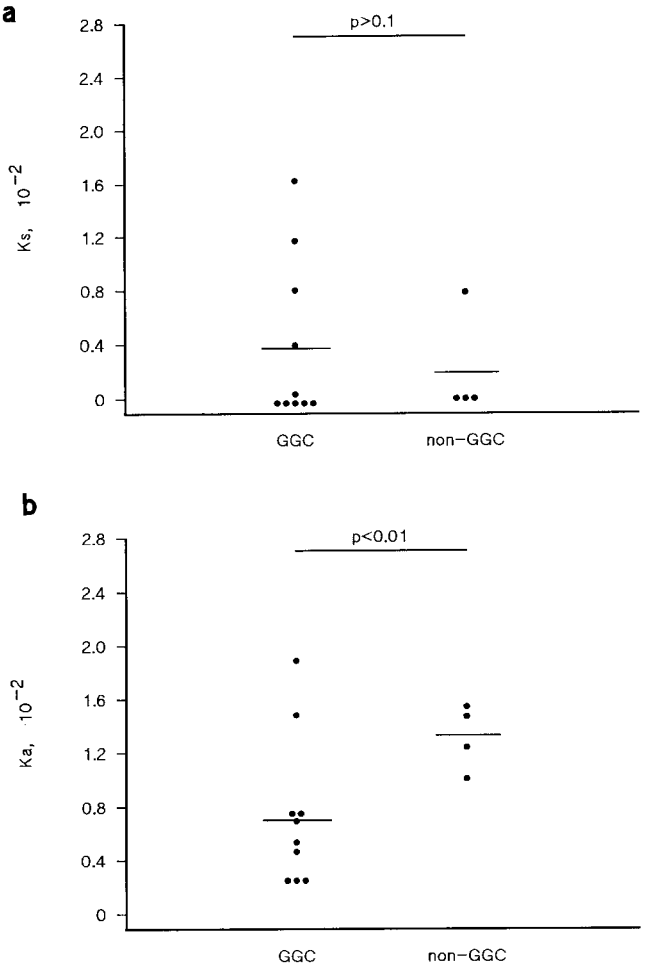

FIG. 4. Numbers of synonymous (a) and nonsynonymous (b) nucleotide substitutions per site per year within the V3 loops (105 nucleotides in length) of HIV-1 strains present in IVDUs infected by HIV-1 strains with and without the GGC signature pattern. Horizontal lines indicate means.

The most striking finding of this study is that HIV-1 strains present in AIDS-free homosexual men accumulated over 5 years significantly more nonsynonymous substitutions within the V3 loop than HIV-1 strains in AIDS-free IVDUs. This phenomenon was a result of lower rates of nonsynonymous evolution in IVDUs in both the initial 3-year period from seroconversion and the following 2-year period, as well as a larger proportion of nonsynonymous back substitutions in IVDUs.

The route of virus transmission per se together with the size of transmission bottleneck may be the cause of the relatively slow evolution in IVDUs. If we assume that a lower evolution rate reflects faster adaptation of virus to the host, at the same time it indicates higher initial fitness of the virus population in its individual environment. In IVDUs this might be the case. A larger infectious dose and direct presentation of virus to $CD4^+$ lymphocytes could result in a fast adaptation and relative evolutionary stasis of the transmitted HIV-1 population. Conversely, in homosexual men a considerable bottleneck might be operational; most viruses that are transmitted may exhibit suboptimal fitness, and therefore advantageous nonsynonymous substitutions are accumulated, resulting in increasing virus fitness.

The lower initial fitness of viruses in homosexual men relative to viruses in IVDUs could be the result of at least two processes: (i) random transmission of virus variants with low fitness and/or (ii) the initial adaptation of the virus population to target cells other than the final virus-producing cells. The genomic region analyzed in our study (the V3 loop) carries determinants of HIV-1 cell tropism (6, 7), and therefore HIV-1 strains optimally adapted to certain cells may exhibit lesser adaptation to another cell type. This hypothesis is supported by evidence for virus compartmentalization in the host, including genetic distinctions between HIV-1 population present in blood and semen (40) and in blood and intestinal tissue (2, 32, 33), as well as by the findings of Novella et al. (29) and Duarte et al. (11), who observed a decrease of virus population fitness during low-dose in vitro passage of the vesicular stomatitis virus.

Since the HIV-1 strains in Dutch IVDUs can be genetically distinguished from those in homosexual men (16–18, 22), greater fitness or better adaptation and, as a result, the slower intrahost V3 evolution in IVDUs may be related to the inherent properties of these distinct HIV-1 subpopulations. There may be a lineage of virus that retains specific biological properties and is transmitted preferentially among IVDUs. A recent study revealed that in addition to the substitutions in the V3 region, HIV-1 strains in Dutch IVDUs have a number of nucleotide substitutions in *env*, *vpr*, and *vpu* genes compared to HIV-1 strains in homosexual men (16). Since some of these substitutions are nonsynonymous, these multigenic differences in HIV-1 strains of homosexual men and IVDUs may have functional significance (16). This hypothesis is in accord with our observations that the population-wide evolution of HIV-1 is slower in the Amsterdam cohort of IVDUs than in homosexual men (18, 20a). We attempted to test the hypothesis that inherent properties of the viruses are related to the difference in evolution rates by comparing the HIV-1 evolution in IVDUs infected by HIV-1 strains with and without the GGC triplet. Since the GGC pattern is highly conserved in IVDUs in Amsterdam, we were unable to find seroconvertors among IVDUs infected by HIV-1 strains without this marker who were AIDS free for at least 5 years. Therefore, we added another 14 seropositive IVDUs to the study. Viruses of four of them lacked the GGC marker and phylogenetically were closely related to viruses of homosexual men. Our observation of a slower intrahost evolution in IVDUs infected by the HIV-1 strains with the GGC marker is in accord with the hypothesis that the founder HIV-1 population influences HIV-1 evolution. However, since an overlap between risk groups of IVDUs and homosexual men as well as risk factors other than intravenous drug injection cannot be ruled out, it is hard to prove that IVDUs harboring HIV-1 populations lacking the GGC marker were not infected by male-to-male or heterosexual HIV-1 transmission. In this case, the observation of a slower intrahost evolution in IVDUs infected by the HIV-1 strains with the GGC marker provides independent evidence for the influence of transmission bottlenecks on HIV-1 evolution in a new host.

Finally, since the intrahost evolution of HIV-1 has been associated with immune competence of the host (21), an alternative explanation of the slower evolution of the V3 loop in IVDUs could be provided by the quality of the adaptive immune response in IVDUs. Although homosexual men and IVDUs were matched for $CD4^+$ cell count in our study and, in accord with our previous data (21), no correlation was seen between $CD4^+$ cell counts and HIV-1 evolution in immunocompetent hosts, it is still possible that IVDUs exhibit weaker immune surveillance due to functional abnormalities of $CD4^+$

cells (rather than to their numbers) or to another factor(s) yet unidentified. Drug abuse has been associated with immunological abnormalities such as hypergammaglobulinemia (mainly involving immunoglobulins M and G) and defects of cellular immunity (impaired lymphocyte reactivity to mitogens, increased numbers of atypical lymphocytes, etc.) (1, 3, 5, 10, 23, 35). The HIV-1 target cells (T cells and monocytes) carry receptors for opiates (24, 38). The proliferative response after stimulation with anti-CD3 monoclonal antibodies is significantly decreased in IVDUs who frequently inject drugs (25). Unfortunately, we do not have sufficient data to demonstrate a difference in anti-HIV immune response in IVDUs compared to other risk groups. Moreover, despite differences in the course of HIV-1 infection between homosexual men and IVDUs, the rate of disease progression, which is often used as a measure of the ability of the host to control virus infection, is similar in the two risk groups. Nonetheless, the higher K_s/K_a ratios suggest that the viruses in IVDUs are subjected to less selection pressure after the transmission event.

Since intrahost evolution of HIV-1 is a result of complex interactions between host and virus, all mentioned factors together may contribute to the difference in HIV-1 evolution between risk groups. In IVDUs, primary infection may be established by HIV-1 strains that exhibit optimal fitness due to larger infectious dose, direct presentation of virus to $CD4^+$ lymphocytes, and/or inherent properties of the virus population. High initial fitness of HIV-1 strains in IVDUs can explain the low rate of evolution and a large proportion of nonsynonymous back substitutions in this risk group. At the same time, weaker immune surveillance in IVDUs could allow this originally present virus population to be preserved or restored.

In conclusion, in this study we attempted to test whether direct contact of an HIV-1 donor population with $CD4^+$ lymphocytes following parenteral HIV-1 transmission results in a difference in HIV-1 intrahost evolution compared to indirect presentation of a virus donor population by sexual (male-to-male) transmission. We obtained preliminary evidence that under similar clinical and immunological conditions, the route of virus transmission and/or the founder virus population may influence the intrahost HIV-1 evolution. Based on the restriction to the V3 loop and lower statistical significance, this influence, however, is considered less strong than that of the intrahost environment (21).

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