Absence of HIV-1 Superinfection 1 Year after Infection between 1985 and 1997 Coincides with a Reduction in Sexual Risk Behavior in the Seroincident Amsterdam Cohort of Homosexual Men

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Background. Incidence rates of human immunodeficiency virus type 1 (HIV-1) superinfection differ among cohorts and, as yet, only 2 cohorts of homosexual men have been screened. Here, we investigated the incidence of HIV-1 superinfection during the first year after infection among homosexual participants in the Amsterdam Cohort Studies on HIV infection and AIDS who seroconverted between 1985 and 1997.

Methods. We analyzed env C2–C4 diversity in the serum of therapy-naive participants, using a heteroduplex mobility assay; heteroduplexes were considered to be indicators of potential dual infections, in which case env C2–C4 polymerase chain reaction (PCR) products were cloned and sequenced. Sequences were subjected to phylogenetic analysis. Data on the sexual behavior of participants were collected from 1 year before seroconversion until the end of the investigated period.

Results. For 89 seroconverters with a detectable viral load (>1000 copies/mL), env PCR products were generated from serum samples obtained at seroconversion and 1 year later. Heteroduplexes were observed in 68 of the 89 patients; among these 68 patients, a median of 9 molecular clones per time point was sequenced. Phylogenetic analysis did not reveal evidence for superinfection; 1 patient was HIV-1 coinfected. Shortly after diagnosis of HIV infection, the number of sex partners decreased, the frequency of anal intercourse declined, and condom use increased.

Conclusions. The incidence of HIV-1 superinfection soon after seroconversion in this cohort is low. Risk reduction shortly after HIV-1 diagnosis early during the HIV-1 epidemic in the Netherlands may have contributed to the absence of HIV-1 superinfection observed in this study.

Natural immune responses after primary human immunodeficiency virus type 1 (HIV-1) infection have been thought to protect against HIV-1 superinfection in vivo [1, 2]. However, as early as 1995 dual infections were reported in patients for whom superinfection could not be excluded [3–5]. Dual infection comprises coinfecion and superinfection, with coinfecion defined as (nearly) simultaneous infection with multiple viral strains at or around seroconversion and superinfection defined as the acquisition of another viral strain after the development of an immune response to the initial infection [6]. Since 2002, >20 publications have reported close to 50 well-documented cases of HIV-1 superinfection [7–26], including 2 cases of triple infection with HIV-1 [27, 28]. Because the HIV-specific immune response develops during the course of infection, individuals with acute HIV-1 infection are considered to be more susceptible to superinfection than chronically infected individuals. Overall, approximately half of the published cases...
of superinfection were acquired within ~1 year after seroconversion [9, 10, 12, 15–24]. However, we recently reported the occurrence of superinfection in a long-term elite controller >13 years after primary infection [29], indicating that mechanisms that halt disease progression may not be able to protect against superinfection.

The rate of HIV-1 superinfection remains a matter of controversy, with reported rates being close to the rate of initial infection in some studies [15, 17, 22, 24] and superinfection being absent in others [30, 31]. Commercial sex worker cohorts have mostly been screened for superinfection, which has resulted in the detection of almost half of all published cases of superinfection [13, 16–18, 22, 24]. In addition, 4 injection drug user cohorts have been studied [9, 11, 20, 30], revealing 7 cases. In 2 studies, cohorts of men who have sex with men have been screened for superinfection [14, 15], revealing a total of 4 cases.

In the present exploratory study, HIV-1–positive therapy-naive homosexual male participants in the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS) who had documented seroconversion dates between 1985 and 1997 were screened for HIV-1 superinfection 1 year after seroconversion, and sexual behavioral data for the periods before and after seroconversion were analyzed.

**METHODS**

**Patient population and sample selection.** ACS investigations are conducted in accordance with the ethical principles set out in the Declaration of Helsinki. Enrollment began in October 1984; through 31 December 2008, 2383 participants have had at least 1 visit, with 1588 testing seronegative for HIV, 585 testing seropositive for HIV, and 210 experiencing HIV seroconversion. Clinical and epidemiological data are collected, CD4 T cell counts and plasma viral loads are determined, and serum and peripheral blood mononuclear cells are obtained and stored at 3-month intervals. Written informed consent is obtained from every participant.

**RNA isolation and amplification of HIV-1 env C2–C4 by reverse-transcription polymerase chain reaction.** Viral RNA was isolated from 140 µL of serum (QIAamp Viral RNA Mini kit; Qiagen) and eluted in 50 µL. Ten microliters of viral RNA containing a median of 1386 RNA copies (range, 56–728,000 RNA copies) was reverse transcribed (SuperScript First-Strand Synthesis system; Invitrogen) into complementary DNA, using the sequence-specific primer Seq2 (5′-TCTCCATATCTCTCCTCCTCCAGGTTC-3′) and Seq3 (5′-TATGGGATCAAGGCTTAACGGAT-3′). Five microliters of complementary DNA was subjected to first-round polymerase chain reaction (PCR) in a volume of 25 µL (primers, Seq2 and Seq3 [5′-TATGGGATCAAGGCTTAACGGAT-3′]). Two microliters of first-round PCR product was subjected to second-round PCR (primers, Seq5 [5′-GTCAACTCAGTGGTGTTTATGTC-3′] and Seq6 [5′-ATCTAATTTGTCACCTGATGGGAGG-3′]) in a volume of 25 µL, generating a 549-nucleotide fragment of env C2–V3-C3–V4-C4 (HXB2R positions 7012–7560). Amplification conditions for both first- and second-round PCR were as follows: 1 cycle at 94°C for 5 min; 35 cycles at 94°C, 50°C, and 70°C for 45, 30, and 90 s, respectively; and a final step at 70°C for 10 min.

**Heteroduplex mobility assay of HIV-1 env C2–C4.** Heteroduplexes were generated by denaturing 5 µL of second-round PCR product from each time point separately and a mix of PCR products from the 2 time points for each patient at 95°C for 2 min in 10× heteroduplex annealing buffer [32]. Specimens were immediately transferred to wet ice, allowing the formation of homo- and heteroduplexes within the quasispecies of patients’ env sequences, and resolved on a 5% non-denaturing polyacrylamide gel.

**Molecular cloning and sequencing of HIV-1 env C2–C4.** Second-round PCR products were cloned into the pGEM-T Easy Vector system (Promega), transformed into competent DH5α Escherichia coli (Invitrogen), and plated on Luria-Bertani agar with blue-white screening. White colonies were picked at random (4–32 colonies per reaction). Cloned PCR products were amplified (vector primers, T7 [5′-TAATACGACTCACTATAGGGG-3′] and SP6 [5′-GATTATAGGTAGACTATAG-3′]) using the above-described PCR program. After purification of PCR products (ExoSAP-IT; USB), the T7 and SP6 primers were used for sequencing (Big Dye Terminator Cycle Sequencing kit, version 1.1; Applied Biosystems). Sequences were determined using an automated DNA sequencer (Applied Biosystems). When sequence diversity did not reflect the heteroduplex pattern obtained by heteroduplex mobility assay (HMA), an additional 4 nested PCRs were performed, and products were cloned and plated. Two clones per plate were picked, PCR with the SP6 and T7 primers was performed, and PCR products were sequenced.

**Sequence analysis.** Clonal sequences were aligned for each patient by means of the ClustalW algorithm [33], and alignments were manually edited using BioEdit software (BioEdit, version 7.0.5.3; Ibis Biosciences). Alignments were visually inspected for the presence of mismatches, insertions, and deletions.

**Phylogenetic analysis.** For phylogenetic analysis, all patients’ alignments were merged. Published sequences from samples isolated in the Netherlands were downloaded (http://www.hiv.lanl.gov) to serve as a local control panel. Additionally, a similarity search was performed using BLAST software [34] to retrieve the sequences most similar to those generated in the present study. This panel of highly related but epidemiologically unlinked sequences was merged with the local control panel and the merged patients’ alignments. The resulting alignment was subjected to the ClustalW algorithm and manually edited. The corresponding nucleotide substitution model was chosen.
with Modeltest, version 3.7 [35], using hierarchical likelihood tests. Subsequently, phylogenetic analyses were performed using PAUP* software, version 4.0 [36]. A neighbor-joining tree was inferred followed by a heuristic search for a maximum-likelihood (ML) tree, making use of the best-fit substitution model starting with the neighbor-joining tree. Statistical support for nodes was generated with bootstrapping on the neighbor-joining tree (1000 repeats). The maximum-likelihood tree was rooted with a non–B subtype sequence.

**Sexual risk behavior.** Behavioral data were collected from structured questionnaires administered at 6-month intervals, representing behavior in the preceding 6 months. To investigate changes in sexual behavior after seroconversion, we summarized self-reported sexual risk behavior among study participants during the 12 months before seroconversion and from seroconversion onward until the second time point analyzed. Sexual behavior for the present study consisted of the median number of male sex partners, the median number of insertive and receptive anal sex partners, and inconsistent condom use during insertive and receptive anal intercourse. Differences in sexual behavior before and after seroconversion were tested by the Wilcoxon signed rank test for the median number of male sex partners and insertive and receptive anal sex partners per period and by generalized estimating equation logistic regression (univariate) for inconsistent condom use (SPSS statistical software package, version 15).

**RESULTS**

**Study cohort and selected samples.** In total, 141 homosexual men participating in the ACS who had a documented seroconversion date between 1985 and 1997 (median date, 1987) (Figure 1) were screened for availability of serum samples with a detectable viral load (>1000 copies/mL of plasma) close to seroconversion and 1 year later. For 46 patients, viral load was undetectable or serum samples were not available at either the first or second time point, leaving 95 patients for analysis (Figure 2). The first time point was at a median of 2 months after seroconversion (range, 4 months before to 8 months after seroconversion), and the second time point was at a median of 14 months after seroconversion (range, 8–19 months after seroconversion). The median difference between the 2 time points was 12 months (range, 6–21 months). PCR fragments of *env* C2–C4 from both time points could be generated for 89 of 95 patients and were studied for heterogeneity by HMA (Figure 2).

**Heterogeneity in env C2–C4 as detected by HMA and sequence analysis.** Heteroduplexes of *env* C2–C4 PCR fragments generated from each patient’s serum sample obtained close to seroconversion or 1 year later and/or additional heteroduplexes in the mixture of the PCR fragments were considered to be an indication of a possible dual infection in a patient. In PCR products from serum samples from 21 patients, heteroduplexes were not observed (Figure 2). Because low copy numbers may account for the absence of heteroduplexes, serial end-point dilution PCR was performed on samples from all 21 homoduplex-only patients. For these 21 patients, the number of analyzed *env* fragments was indeed too low (median, 2.92 copies/5 μL of input in the first-round PCR; range, 0.35–9.88) to firmly exclude the presence of dual infection. Heteroduplexes were observed in PCR products from 68 of 89 patients (Figure 2). To determine whether these heteroduplexes were the result of dual infection, second-round PCR products from both time points for these 68 patients were cloned, and 4–32 colonies per time point were sequenced. If sequence variability (mismatches or insertions and deletions in sequence alignments) did not match the HMA heteroduplex pattern, another 4 nested PCR products were generated and cloned, and 2 colonies per cloning reaction were picked and sequenced. Sequences were added to the initial alignments, and sequence variability was again compared with the HMA. After this procedure, both methods (HMA and molecular cloning followed by sequencing) gave concordant results. In total, 1261 sequences were kept for phylogenetic analysis (median, 9 sequences per time point; range, 2–22). Visual inspection of alignments indicated the presence of 2 distinct viral strains in 1 patient at the first time point, indicating HIV-1 coinfection.

**Demonstration by phylogenetic analysis of the absence of HIV-1 superinfection.** The merged patients’ alignments were combined with a reference set comprising 264 local control sequences and 242 highly similar globally sampled sequences. The neighbor-joining tree was constructed using PAUP* soft-
Figure 2. Approach for patient selection and identification of human immunodeficiency virus type 1 (HIV-1) superinfection among homosexual seroconverters in the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS). Patients were screened for available serum samples with a detectable viral load (>1000 copies/mL) at around seroconversion and ~12 months later. Samples were subsequently analyzed by heteroduplex mobility assay (HMA) on polymerase chain reaction (PCR) products of the env C2–C4 region. Viral copy number was determined by limiting-dilution PCR for patients whose samples showed the absence of heteroduplexes by HMA. Samples from the other patients were further analyzed by clonal sequencing and phylogenetic analysis. CI, confidence interval; PYs, person-years; SC, seroconversion; TPs, time points.

Figure 3. Maximum likelihood tree of env sequences generated from serum samples obtained at around seroconversion and ~1 year later for 68 homosexual seroconverters in the Amsterdam Cohort Studies on HIV Infection and AIDS.

The incidence of initial HIV-1 infection and HIV-1 superinfection in the ACS. The yearly incidence rate of initial HIV-1 infection among homosexual men participating in the ACS between 1985 and 1997 ranged from 0.23 to 7.59 cases per 100 person-years (PYs) (Figure 1), with an overall incidence rate during this period of 2.25 cases per 100 PYs (95% confidence interval [CI], 1.9–2.7). The majority of our study participants seroconverted between 1985 and 1987, with yearly incidence rates of 7.59, 3.83, and 2.58 cases per 100 PYs, respectively, and an overall incidence rate of 4.45 cases per 100 PYs (95% CI, 3.5–5.5) (Figure 1). The absence of HIV-1 superinfection among 68 homosexual men in the ACS resulted in an overall HIV-1 superinfection incidence rate of 0 cases per 100 PYs (95% CI, 0–5.0) (Figure 1). Although we did not detect HIV-1 superinfection, given the overlapping 95% CI we cannot assume that the incidence rates of the initial HIV-1 infection and the subsequent HIV-1 superinfection are statistically significantly different in this group.

Decline in sexual risk behavior after diagnosis of HIV infection. We next wanted to explore reasons for the absence of superinfection in the ACS 1 year after infection. Self-reported behavioral data were available from 243 questionnaires for 86 of 89 seroconverters in the periods before seroconversion and after seroconversion until the second time point investigated. Available data from before and after seroconversion on 72 seroconverters were included in the analysis of the median number of sex partners. The median number of total sex partners significantly declined from 12.3 (interquartile range [IQR], 4.5–24.4) during the 12 months before seroconversion to 6.0 (IQR, 1.6–15.4) during the period after HIV diagnosis (P < .001) until the second time point investigated in this study (Table 1). After HIV diagnosis, the median number of insertive and receptive sequences from both time points together in 1 cluster per patient (see the maximum-likelihood tree in Figure 3). As an exception, 1 patient appeared to be HIV-1 coinfected: sequences from the first time point were retrieved in 2 distinct clusters, one of which also contained the sequences from the second time point (see patient 61 in the maximum-likelihood tree in Figure 3). Hence, the patient was infected with 2 distinct viral strains at or around seroconversion, of which one was detectable at seroconversion but was undetected at the second time point. All HIV-1 sequences from study participants were subtype B.

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Inconsistent condom use during receptive anal intercourse declined from 37.1% to 29.2% (odds ratio, 0.68; 95% CI, 0.37–1.26), but this decrease did not reach statistical significance ($P = 0.21$). Inconsistent condom use during receptive anal sex did significantly decline, from 56.9% to 33.9% (odds ratio, 0.35; 95% CI, 0.21–0.58; $P = 0.001$) (Table 2). Overall, unsafe sexual behavior significantly declined after the participants became aware of their HIV-positive status. Because the 68 participants for whom superinfection could be ruled out might constitute a subgroup, we also performed the analysis of the behavioral data for this group only. Data were available for 65 of the 68 participants and yielded the same results as those described above, but with slightly higher $P$ values (data not shown).

**DISCUSSION**

In the ACS, we observed a low incidence rate of HIV-1 superinfection during the first year after seroconversion (yearly incidence rate, 0 cases per 100 PYs; 95% CI, 0–5.0) among homosexual men during the period between 1985 and 1997. To our knowledge, this is the first report that links the absence of HIV-1 superinfection with a decline in sexual risk behavior. HIV-1 *env* C2–C4 sequence heterogeneity in the serum of cohort participants was prescreened by HMA, multiple molecular clones for 68 of 89 patients were sequenced, and no evidence of superinfection was found; 1 case of HIV-1 coinfection was observed. For 21 of 89 patients, superinfection could not be ruled out because the input of DNA genome copies was too low.

Similar to other studies, ours may underestimate the actual incidence of superinfection. First, we analyzed only 2 time points per patient, targeting the first year after seroconversion; transient superinfections and/or superinfections after the first year of infection were therefore not detected. Second, recombinant strains differing in genomic parts other than the *env* C2–C4 region were not detected. Finally, superinfecting strains constituting <1.4% variation relative to any other viral strain within the viral quasispecies remained undetected because of the threshold of genetic difference for generation of HMA heteroduplexes [37]. However, our approach is equivalent to those of other studies that did report superinfection [10, 12, 14–16, 18, 27], given that we applied HMA and clonal sequencing instead of population sequencing, which is less sensitive for detecting minor strains [37]. Although samples were from the early stages of the Dutch HIV epidemic, HIV-1 variants circulating then were sufficiently different to allow discrimination between variants from any 2 investigated individuals in the cohort (minimum genetic distance between patients, 3%; data not shown).

An explanation for the absence of superinfection could be the level of risk behavior. Half of all documented superinfection cases have been identified via screenings of female cohorts at high risk for HIV-1 infection [13, 16–18, 22, 24]. Risk behavior has been reported as 1–2 sex partners per week [22], and for 2 superinfected women 3 and 30 sex partners per week, respectively, were described [16]. Our cohort, however, reported a median of only 6 sex partners per participant during the 12 months after seroconversion, implying a 10–30-fold lower number of sex partners compared with that among these female sex worker cohorts.

**Table 1. Number of Total and Anal Sex Partners for Study Participants Before and After Seroconversion (SC)**

<table>
<thead>
<tr>
<th>Category</th>
<th>Before SC</th>
<th>After SC</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sex partners$^b$</td>
<td>12.3 (4.5–24.4)</td>
<td>6.0 (1.6–15.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insertive anal sex partners</td>
<td>3.0 (1.0–7.0)</td>
<td>1.0 (0.0–4.0)</td>
<td>0.007</td>
</tr>
<tr>
<td>Receptive anal sex partners</td>
<td>1.5 (0.5–4.8)</td>
<td>1.0 (0.0–3.0)</td>
<td>0.038</td>
</tr>
</tbody>
</table>

**NOTE.** Information was available for both time points from 72 of the 89 study participants. IQR, interquartile range.

$^a$ By the Wilcoxon signed rank test.

$^b$ Also includes nonanal sex partners.

**Table 2. Inconsistent Condom Use during Anal Intercourse for Study Participants Before and After Seroconversion (SC)**

<table>
<thead>
<tr>
<th>Inconsistent condom use</th>
<th>Before SC</th>
<th>After SC</th>
<th>OR (95% CI)</th>
<th>$P^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>During insertive anal intercourse</td>
<td>26/70 (37.1)</td>
<td>28/96 (29.2)</td>
<td>0.68 (0.37–1.26)</td>
<td>0.21</td>
</tr>
<tr>
<td>During receptive anal intercourse</td>
<td>66/116 (56.9)</td>
<td>41/121 (33.9)</td>
<td>0.35 (0.21–0.58)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

**NOTE.** Of the 89 study participants, information was available for 74 men with 166 observations for insertive anal sex and for 86 men with 237 observations for receptive anal sex. CI, confidence interval; OR, odds ratio.

$^a$ From univariate generalized estimating equation logistic regression.
Here, we report the absence of HIV-1 superinfection in a cohort of homosexual men early during the Dutch HIV epidemic. A low incidence rate was also reported in a superinfection screening of a US cohort of homosexual men, with 1 case of superinfection detected by analyzing at least 4 samples from 32 participants in the Multicenter AIDS Cohort Study at 6-month intervals [14]. In another US cohort, Smith et al [15] detected 3 cases of superinfection among 76 homosexual men in samples spanning the first 6–12 months after seroconversion (incidence rate, 5.0 cases per 100 PYs; 95% CI, 1.7–13.3) between 1997 and 2004, a period during which highly active antiretroviral therapy became widely available in high-income countries and the resurgence of unprotected anal intercourse with more partners was reported.

At the beginning of the HIV epidemic, safer sex strategies, such as consistent condom use during anal intercourse and a reduction in the number of sex partners, were greatly promoted to halt HIV-1 transmission, and these risk-elimination messages resulted in a decline in HIV-1 transmission rates in homosexual communities [38, 39]. Interestingly, the absence of HIV-1 superinfection in our cohort during the first year of HIV-1 infection coincided with a statistically significant reduction in sexual risk behavior after being given a diagnosis of HIV-1 infection. Being aware of their HIV-positive status, our study participants reported significantly fewer total and anal sex partners and significantly more frequent condom use. As a result of early preventive activities in the Netherlands, the number of partners also declined among seronegative participants, but the reduction was far more pronounced among seroconverters after diagnosis of HIV-1 infection [38]. All these behavioral changes are described in the context of risk-elimination messages early during the HIV-1 epidemic. In a case-control study, seroconverters in the ACS were more likely to have had more sex partners—specifically, more partners for receptive anal intercourse—than control subjects [40]. The overall HIV-1 incidence rate over the period between 1985 and 1997 was 2.25 cases 100 PYs (95% CI, 1.9–2.7) in the homosexual male cohort of the ACS (the yearly incidence rates ranged from 0.23 to 7.59 cases per 100 PYs) (Figure 1). However, the majority of study participants seroconverted in 1985, 1986, and 1987 (Figure 1), with yearly HIV-1 incidence rates of 7.59, 3.83, and 2.58 cases per 100 PYs, respectively (overall HIV-1 incidence rate, 4.45 cases per 100 PYs; 95% CI, 3.5–5.5).

The incidence of HIV-1 superinfection most likely depends on the number of unprotected sex acts and sex techniques, but it also depends on the HIV-1 prevalence within sex networks. Given that the prevalence of HIV-1 infection was high among homosexual men in Amsterdam early during the epidemic (estimated HIV-1 prevalence between 1985 and 1987, 31%–39%), behavioral factors not related to HIV-1 prevalence are more likely to have played a role in our cohort [39]. Further research is required to elucidate the effect of each factor on the acquisition of HIV-1 superinfection.

In conclusion, the absence of persisting superinfection in our study cohort may be related to the low level of risk behavior after seroconversion as a consequence of risk-elimination messages early during the HIV epidemic. Studies of seroconverter cohorts with substantial follow-up, in which continuous and/or increased sexual risk behavior with a prolonged time since HIV-1 diagnosis might occur, are needed to precisely estimate the incidence of superinfection among homosexual men who do not reduce sexual risk behavior after receiving a diagnosis of HIV-1 infection.

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Potential conflicts of interest. All authors: no conflicts.

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