Silent Human Immunodeficiency Virus Type 1 Infection: A Rare Occurrence in a High-Risk Heterosexual Population


A group of 58 heterosexual female partners (FP) of human immunodeficiency virus type 1 (HIV-1)-seropositive hemophiliacs was studied by conventional diagnostic methods such as enzyme-linked immunosorbent assay (ELISA) and Western blot analysis to examine whether any had acquired HIV-1 infection through sexual transmission. A subset of 29 FP were asked to answer a detailed questionnaire concerning their health, use of “safer sex” techniques, and other risk factors for HIV-1 infection. They also had additional blood drawn for CD4 cell analysis, viral cultures, nef, gag, and env immunobots, and polymerase chain reaction (PCR) analysis to assess the occurrence of “silent” HIV-1 infection in a high-risk seronegative population. Among the 58 FP, three were found to be HIV-1-seropositive on first testing, with no new seroconversions occurring with subsequent testing in the remaining 55. Two seropositive FP had the additional testing and were found to have positive viral cultures, as well as positive PCR results. All of the seronegative FP (n = 24) who had additional testing were negative in viral culture, had negative immunobots, and had no HIV-1 nucleic acid sequences detected by PCR. Thus, in this population, silent HIV-1 infection appears to be a rare occurrence and antibody testing seems to correlate with the more sensitive techniques of PCR and viral cultures.

HETEROSEXUAL transmission of human immunodeficiency virus type 1 (HIV-1) has been well documented in many studies. Female sexual partners (FP) of men in high-risk groups for HIV-1 infection themselves represent a risk group for acquiring infection. The hemophilic population represents an excellent model for studying heterosexual transmission. Hemophiliacs were infected with HIV-1 from the late 1970s through 1984 secondary to infusion with coagulation factor concentrate made from contaminated plasma. Most hemophilic males were thus already infected with HIV-1 when serologic testing became widely available. The use of viral inactivated concentrates in 1985 has resulted in a marked decrease in new HIV-1 infections in this cohort. The heterosexual transmission rate in this group has been quoted in ranges from 7% to 22% depending on which cohort and what area of the United States has been studied. Many of the FP of hemophiliacs have been exposed to HIV for years through unprotected intercourse with their seropositive mate, since testing for HIV-1 in hemophiliacs began widely only in 1985. Because of prolonged exposure, this group may be at risk for “silent” HIV-1 infection (infection in the absence of an antibody response).

It is still not clear what causes HIV-1 to become productive once it has infected the host cell. Various factors including other viral infections can activate latent HIV-1 in cells. However, once the switch is made from latent to productive viral replication, the host immune mechanism responds to the infection. Seroconversion to HIV-1 in persons exposed to HIV-1 has been reported to occur within 3 to 8 weeks after the initial exposure. However, in certain at risk cohorts, HIV-1 has been detected in lymphocytes many months before actual seroconversion occurred. In one study of isolation of HIV-1, polymerase chain reaction (PCR) was found to be positive in nine of 26 seronegative FP of hemophiliacs studied. Silent infection has also been reported in a group of 133 seronegative homosexual men engaging in high-risk behavior. Of this group, 23% were found to have positive viral cultures, although maintaining a seronegative state for up to 36 months. In another homosexual cohort, the HIV-1 genome was detected by PCR using both HIV-1 gag and env primers in 20 of 24 people at a median of 18 months before seroconversion occurred. PCR positivity and antibody response were concurrent in only four of the individuals. These studies suggest that a long latency period between HIV-1 infection and triggering of an immune response may occur in some HIV-1-infected individuals. In reanalyzing their data, Imagawa and Detels have suggested a second hypothesis, that of “incomplete infection” to explain their initially positive PCR data. In contrast, more recent studies have shown that detection of the HIV-1 genome long before the appearance of serologic evidence of infection is rare in high-risk but seronegative individuals or in seronegative heterosexual FP of seropositive hemophiliacs.

To examine whether silent HIV-1 infection occurs in FP of hemophilic partners who engaged in unprotected intercourse, a cohort of heterosexual FP of seropositive hemophiliacs was studied with viral cultures and PCR analysis.

MATERIALS AND METHODS

Patients. Fifty-eight FP of HIV-seropositive hemophilic males followed at the New England Hemophilia Center were screened for the presence of HIV-1 antibody. The FP were tested only after signing a detailed consent form and receiving extensive pretest and posttest counseling. Their median age was 30 years (range, 19 to 72 years). All were tested from 1 to 5 years after seroconversion was identified in their male partner. Thirty-five FP had repeat follow-up HIV-1 testing at least twice (range, 2 to 11 times). Seventeen of their hemophilic partners had acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC), and five were HIV-seronegative.
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...ency syndrome (AIDS)/AIDS-related complex (ARC) at the time of testing.

Of these 58 FP, 29 agreed in addition to HIV-1 antibody testing to allow additional testing including CD4 lymphocyte levels, HIV-1 viral cultures, and PCR testing of their peripheral blood mononuclear cells to look for silent HIV-1 infection. The 29 FP who agreed to more detailed studies did not differ in age from the large cohort, 31.1 years (range, 20.4 to 72 years). Of this group, 19 had repeated testing. Four had hemophilic partners with AIDS/ARC, and seven had hemophilic partners with CD4 cell counts less than 200/µL. These FP also agreed to answer a questionnaire concerning their sexual practices and their health status. The forms were coded and confidentiality was assured. A flow sheet of the FP included in each step of testing is seen in Fig 1.

**Methods.** HIV-1 antibody was determined by enzyme-linked immunosorbent assay (ELISA; Abbott, Chicago, IL), and all positives were confirmed by Western blot.

Peripheral blood mononuclear cells (PBMC) were separated from whole blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Chemicals, Piscataway, NJ). T-cell subsets were determined by direct immunofluorescence using fluorescein-conjugated mouse monoclonal antibodies (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and analyzed on a FACS Scan Flow Cytometer (Becton Dickinson). Absolute CD4 lymphocyte counts were calculated by multiplying the percentage times the absolute lymphocyte count. The cells were subsequently frozen using a programmable cell freezer in liquid nitrogen. The mode of collection and storage was uniform for all samples drawn.

**Recombinant HIV-1 nef immunobots.** Western blot assay was performed to detect specific antibodies to HIV-1 nef protein in the serum. It has been suggested that the negative regulatory factor (nef) functions as a suppressor of active viral replication in infected cells. Therefore, it is possible that antibodies to nef protein may be present in the absence of other serological reactivities. In this procedure, recombinant HIV-1 nef protein (NIAID AIDS Reference Repository, Hoffman-LaRoche, Nutley, NJ) was first bound to nitrocellulose papers, which were then treated with nonfat dry milk to block nonspecific binding of proteins. Nef papers were then reacted with the patient sera, followed by colorimetric development using an alkaline phosphatase-conjugated goat anti-human immunoglobulin to test for nef antibodies. Similar procedures were performed using recombinant env and gag recombinant proteins.

**HIV-1 culture.** PBMC isolated from the FP were cultured in vitro using the consensus technique developed by the Viral Research Laboratory (VRL) of the ACTG network. To activate and enhance viral replication, the cells were cocultured with phytohemagglutinin-activated (3 days) normal HIV-1-seronegative PBMC in the presence of 10% interleukin-2 (Pharmacia Chemicals). The cultures were maintained up to 4 weeks and the culture supernatants were sampled twice a week to assay for the presence of HIV-1 p24 gag protein using a commercially available antigen capture ELISA kits (Abbott [Billerica, MA], Coulter [Hialeah, FL]). The culture was considered positive for the viral antigen by comparing the absorbance of the specimen at 492 nm to a cut-off value. If the amount of p24 exceeded 400 pg/mL anytime during the course of the culture or was greater than 30 pg/mL for two consecutive samplings, the specimen was considered positive for the virus. Our laboratory has successfully used this technique to recover HIV-1 from greater than 98% of HIV-1–infected individuals both in adult and pediatric cases.

**PCR for presence of HIV-1 genome.** The PCR was run according to a modification by Saiki et al. Each sample was amplified separately using primers specific to both the HIV-1 env (SK38/SK39) and HIV-1 gag (SK68/SK69) promoter regions. Cells lysate equivalent to 1 µg of genomic DNA was directly amplified in a 50-µL final volume of PCR mixture. The samples were overlaid with 50 µL of mineral oil and then amplified in a thermal cycler machine (Ericon, San Diego, CA). The denaturation was performed at 95°C for 1 minute, annealing was performed at 61°C for 2 minutes, and the elongation was performed at 72°C for 3 minutes for each cycle. The samples were amplified for 30 cycles, then products extracted with chloroform-isoamyl alcohol mixture and then hybridized under aqueous conditions to a 5'-phosphate labeled oligonucleotide probe specific to the amplified region (SK19 for gag and SK70 for env). The hybridized mixture was analyzed on a 10% polyacrylamide gel electrophoresis, and the specific amplified products were detected by autoradiography (Kodak x-ray film, Rochester, NY). HIV-1–negative and –positive patient samples, as well as in vitro tissue culture samples, are routinely included as internal controls for PCR. The PCR reaction mixture containing the corresponding primer pairs and no-sample DNA is also included in the PCR process to test for any contaminating HIV-1 DNA in the reagents. To ensure the presence of amplifiable DNA, amplification of β-globin sequences was also performed.

**RESULTS**

To date, a total of 58 FP of HIV-seropositive males have been tested for HIV-1 antibody, with 60% having repeat testing. Of the 58 FP originally tested, three were found on their first test to be HIV-seropositive (one in July 1989, one in October 1989, and one in June 1986). None of the three hemophilic males were symptomatic for HIV disease at the time their partners were found to be seropositive. Two of the three FP were long-term sexual partners (> 10 years) of the hemophilic male and their exact dates of seroconversion are not known. The third female was a short-term partner and had an additional risk factor of having been a partner of an intravenous drug user. Her date of seroconversion is also unknown.

Of the remaining FP who were initially HIV-seronegative, there have been no seroconversions. On repeated testing, those retested (n = 35) have remained seronegative.
Twenty-nine FP agreed to answer a questionnaire regarding sexual practices. However, as detailed in Fig 1, five of these FP (four HIV-1-seronegative and one HIV-1-seropositive) had incomplete virologic studies and thus are excluded from the study, leaving 24 FP evaluated. The amount of time that these females were sexually active with their HIV-seropositive partner was 147 person-years, which comprised an estimated 10,548 sexual contacts. They reported sexual intercourse a median of 6.5 times per month, with a range of zero to greater than 20 per month. Two of the 24 FP were HIV-seropositive on first testing, one of whom had been abstinent for at least 6 years, and the other who used condoms intermittently. Of the remaining 22 seronegative females, 18 reported using condoms always and three reported using them sometimes since 1986, a much higher percentage than reported by Lusher et al. One couple never used condoms.

Serologic and culture results. Of the FP who answered the questionnaire, 24 had additional viral isolation procedures performed, including two FP who were HIV-1-seropositive. Viral cultures were positive in the two FP who were HIV-1-seropositive and negative in the remaining 22 FP. In both of the FP with positive HIV-1 cultures, ELISA and Western blot were found to be positive. In the 22 FP who were culture-negative, whole virus Western blots, as well as immunoblots using recombinant env, gag, and nef proteins, were negative.

PCR results. The possibility that any of the seronegative FP might be carrying a latent provirus in their cells similar to the observations of Imagawa et al. and Wolinsky et al. was investigated by enzymatically amplifying integrated but potentially latent HIV-1 provirus in host cell DNA. Figure 2 shows the results of PCR using the HIV-1 gag specific oligonucleotide primers, SK38/SK39, followed by liquid hybridization of the amplified product with 32P-end-labeled SK19 oligonucleotide probe. The negative controls for the analysis were uninfected CEM T cells (Fig 2A, lane 1) and PBMCs from normal, seronegative individuals (lane 2). The two known seropositive FP (lanes 3 and 4) and lysates from 8E5 T cells (lanes 10 through 12), which carry a single copy of HIV-1 provirus, were included as positive controls. Lanes 5 through 7 represent lysates from PBMCs of three representative seronegative FP. It is interesting to note that samples in lanes 8 and 9 of Fig 2A represent samples 4 years apart from a seronegative, culture-negative sexual partner. During this 4-year interval, she gave birth to two healthy newborns conceived 2 and 4 years following her seropositive partner’s HIV-1 infection. She still remains seronegative, culture-negative, and PCR-negative. Figure 2C shows that in our routine quantitative PCR analysis (using gag-specific SK38/SK39 primer pairs), we are able to detect between two to four copies of HIV-1 DNA per 1 x 10⁵ cells.

Similar results were obtained when the samples were analyzed by PCR using the HIV-1-specific env primer pairs,
SK68/SK69, and the amplified product was hybridized in solution with the $^{32}$P-end-labeled oligonucleotide probe SK70. Figure 2B represents the specific amplified product in the lysates from the two seropositive FP (lanes 1 and 2) and those from the three seronegative partners (lanes 3 through 5).

The seropositive subjects were PCR-positive and culture-positive, whereas all the seronegative subjects remained PCR-negative and culture-negative. The results are summarized in Table 1. The confidence limit to detect a silent infection is 0% to 14%. In the 22 seronegative FP, the mean CD4 level was 956 ± 375 cells/μL, and the percent CD4 count was 44.2% ± 1.7%, which is not significantly different from a group of normal controls.

**Clinical status of the hemophilic male partners.** Of the 22 hemophilic males associated with the seronegative FP, seven had either a CD4 count less than 200 cells/μL or had AIDS. Eight men were on antiretroviral therapy for CD4 lymphocyte counts less than 500 cells/μL. Four males remain asymptomatic with CD4 counts greater than 500 cells/μL. Eighteen hemophilic males were p24 antigen-negative at the time their FP were tested, three were p24 antigen-positive, and one did not have p24 antigen testing done. The male partners of the two seropositive females (who had complete viral studies performed) were p24 antigen-negative and asymptomatic at the time of the testing of their partners.

**DISCUSSION**

The seroprevalence of HIV-1 antibody positivity in our cohort of FP of HIV-1-seropositive hemophilic males is 5.2%, which is on the lower end of prevalence rates reported for similar cohorts. No new seroconversions have occurred. It is not clear why our seropositivity percentage is low nor why subsequent seroconversions have been absent. By questionnaire, the use of condoms and other forms of "safer sex" is relatively high, but certainly not total.

There also was no evidence of silent HIV-1 infection in the HIV-seronegative FP. All viral isolation procedures, including viral cultures, Western blots, and PCR, were negative when the FP was found to be HIV-1 antibody-negative. Lymphocyte subsets were also normal in the seronegative females. These results are similar to other studies that examined a similar population and found no evidence of silent viral infection in HIV-1-seronegative females. In another study, a very low frequency of silent infection was observed in "at-risk" hemophiliacs. However, one seronegative patient had PCR results that were positive for HIV-1 sequences. No viral cultures were available and it was speculated that laboratory contamination might have been responsible.

Although this study was too small to answer the question adequately, there were no differences in behavior between the females that became HIV-1-seropositive and those that remained negative. One female did have a second risk factor, and one did not use condoms before that time. The immunologic status of the male partners of the seropositive FPs was no different than that of the remaining group. Direct studies of viral burden of the semen may give a better understanding of heterosexual HIV-1 transmission and its predictability.

Based on our results, we would like to suggest that silent infection in HIV-1 pathogenesis, if it occurs, is a rare phenomenon. When a sexually transmitted case of HIV-1 was present in this population, antibody responses were readily demonstrable.

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