RAPID COMMUNICATION

Silent Infections With Human Immunodeficiency Virus Type 1 Are Highly Unlikely in Multitransfused Seronegative Hemophiliacs

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We used the polymerase chain reaction (PCR) to determine the frequency of silent human immunodeficiency virus type 1 (HIV-1) infections in seronegative high-risk individuals with hemophilia who had been exposed to contaminated blood products more than 3 years previously. In a cross-sectional study of a cohort of 57 prospectively followed seronegative hemophiliacs who received multiple transfusions before 1986, HIV-1 proviral DNA was found spontaneously in only one patient. These data suggest that the rate of HIV infection among high-risk antibody-negative individuals with hemophilia is very low to absent, in the range of 0% to 2%. These findings should provide considerable reassurance to seronegative persons with hemophilia and their sexual partners.

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MOST PERSONS with hemophilia who received large amounts of clotting factor concentrates from 1979 to 1985 have developed serologic evidence of infection with the human immunodeficiency virus type 1 (HIV-1).1-5 However, a small minority of individuals treated with factor concentrates during this time have not developed antibodies to HIV-1. The polymerase chain reaction (PCR) is an in vitro method that has been used to selectively amplify specific HIV-1 proviral DNA sequences in peripheral blood mononuclear cells (PBMC) of infected individuals.6 Recently, HIV-1 DNA has been detected by the PCR in PBMC of high-risk seronegative individuals as long as several years before seroconversion.7-9 The proportion and duration of HIV-1 viral infections that escape detection by current serologic tests for HIV-1 antibodies are unknown. However, preliminary data suggest that these controlled or silent infections are more likely to be found in seronegative homosexual men who continue to engage in high-risk behavior than in seronegative hemophiliacs with past exposure to contaminated blood products.10,11

The purpose of this study was to determine the frequency of HIV-1 infections in a cohort of seronegative high-risk individuals with hemophilia who had been repeatedly exposed to contaminated blood products before the implementation of blood donor education, screening, and viral inactivation procedures more than 3 years previously.

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Materials and Methods

Patient population. The Hemophilia Center of Central Pennsylvania located in Hershey provides comprehensive care for over 200 persons with hemophilia who are seen at 6-month intervals for routine evaluation and testing. At each visit, aliquots of sera and PBMC are stored frozen in −70°C and −135°C repositories, respectively. With informed consent, we studied all 57 HIV antibody-negative persons who had ever been exposed to clotting factor concentrates as of September 1989, using frozen PBMC collected at the time of the most recent visit. Forty-two of these individuals had been repeatedly exposed to non-heat-treated factor VIII concentrates before 1986. Fifteen had received only heat-treated concentrates since 1986, but had been repeatedly exposed to fresh frozen plasma or cryoprecipitate before donor screening procedures were implemented in 1985. Serial samples were tested retrospectively on 12 of the most heavily transfused HIV antibody-negative individuals and on four individuals with frozen cells available pre- and posterosconversion. Healthy blood donors and seropositive persons with hemophilia served as negative and positive controls. All specimens were coded and tests were performed in a blinded manner.

Serologic assays. HIV antibody and p24 antigen tests were performed with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Laboratories, Inc, North Chicago, IL), and positive antigen tests were confirmed by neutralization with specific antibody. Western blot analyses were done with the Biotech/Dupont kit (E.I. DuPont de Nemours Co, Wilmington, DE).

HIV-1 cultures. PBMC were separated from whole blood using Lymphocyte Separation Media (Organon Technika, Durham, NC). Ten million patient cells were cocultured with 5 × 10¹ phcytohemagglutinin (PHA)-stimulated donor cells in coculture medium consisting of RPMI-1640 with 15% fetal bovine serum (10%), 5% interleukin-2 (IL-2) (Electronucleonics, Silver Spring, MD), 1% penicillin-streptomycin, and 1% L-glutamine. One-third volume of the culture supernatant was removed every 3 to 4 days and was replaced with an equal volume of fresh coculture medium. Fresh PHA-stimulated normal donor cells were added to the cultures once a week. Aliquots of culture supernatants were tested for HIV p24 antigen using the Enzyme Immunoassay (Abbott). Cultures were terminated when two consecutive HIV p24 antigen determinations ≥30 pg/mL were obtained or when negative results were obtained after 30 ± 2 days.

Enzymatic amplification of HIV-1 DNA. Frozen, density gradient separated PBMC were thawed at room temperature, and washed in phosphate-buffered saline (PBS). Cell lysates for PCR were prepared by resuspending the PBMC pellet in 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 4 mmol/L MgCl₂, 0.5% NP-40, 0.5% Tween-20, and Proteinase K 120 μg/mL. Proteinase K digestion was performed for 1 hour at 55°C. The lysates were then heated to 95°C for 10 minutes to inactivate the Proteinase K. The lysate from 150,000 cells (approximately 1 μg of DNA) was used for each amplification.
reaction. Cell lysates prepared in this manner routinely produced good amplification of PCR primer pairs for a region of DQ or gene.

Enzymatic HIV-1 proviral DNA amplification was performed using PCR essentially as described by Ou et al.12 Primer pairs for conserved regions of the gag and env genes were amplified separately for each sample. The primer pairs used were SK 38-39, which amplifies as 114-bp gag segment (nucleotides 1551 through 1665), and SK 68-69, specific for a 141-bp env fragment (nucleotides 7801 through 7942). Amplifications were performed in 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 200 μM of each deoxyribonucleotide triphosphate, and 2.5 U of Thermus aquaticus (Taq) DNA polymerase (Cetus Corporation, Emeryville, CA). PCR was performed in a Perkin Elmer Cetus DNA Thermocycler for 35 cycles with denaturation at 94°C for 1.5 minutes, primer annealing at 55°C for 1 minute to 32P end-labeled oligonucleotide probes specific for conserved internal amplified sequences of SK 19 (nucleotides 1595 through 1635) for gag or SK 70 (nucleotides 7841 through 7875) for the env PCR product, and template extension at 72°C for 1.5 minutes. The hybridization mixtures were analyzed by 10% nondenaturing polyacrylamide gel electrophoresis, followed by overnight autoradiography with Kodak XAR-5 film (Eastman Kodak, Rochester, NY). The product was electrophoresed in an agarose gel containing ethidium bromide to detect amplified DNA, and the molecular weight of the product was estimated using appropriate markers. Several PBMC lysate specimens from HIV-1 seropositive individuals and low-risk HIV-1 seronegative blood donors were included in each set of assays as positive and negative controls. An aliquot of lysate buffer was amplified concurrently to confirm that the amplification reagents were free of HIV-1 DNA contamination.

For a sample to be considered positive, both gag and env fragments had to be detected by sequential amplification with each primer pair. Samples were scored as negative if either gag or env signal was not detected by the same criteria. Samples with isolated gag or env signals were considered indeterminate and retested. All positive results were confirmed by repeat testing of lysates from separate aliquots of cells. Multiple well-characterized positive and negative controls, including a negative buffer control, were run with each set of experiments.

RESULTS

In a cross-sectional study, HIV-1 proviral DNA was detected in only 1 of 57 (1.8%) of the seronegative recipients of clotting factor concentrates. This individual was an asymptomatic child with a 5% factor VIII level who had received a total of 400 bags of cryoprecipitate from 1981 through December 1987. Two hundred of these bags were given before donor screening was implemented in 1985. Subsequently, he received 15,000 U of heat-treated factor VIII beginning in February 1988. Samples obtained in November 1986 and July 1988 were repeatedly negative. A sample obtained in February 1989 was repeatedly positive using lysates of two separate aliquots of cells. A follow-up sample obtained in March 1990 was again negative, at which time HIV-1 culture of fresh PBMCs was also negative.

HIV cultures were performed on 12 consecutive seronegative patients. All 12 were negative. Twelve intensively treated PCR negative patients had two or more serial samples available for testing over a 1- to 2-year period. HIV-1 proviral DNA was not detected in any of these samples, five of which were also culture negative. Serial samples were also available on four patients before and after seroconversion. HIV-1 proviral DNA was detected in one p24 antigen positive patient before the detection of serum antibody, and in one p24 antigen negative patient 12 months before the detection of serum antibody. HIV-1 proviral DNA was not detected in two other patients 8 and 14 months, respectively, before the appearance of serum antibody. HIV-1 proviral DNA was consistently detected in all 21 HIV antibody positive control individuals, 18 of whom were asymptomatic.

DISCUSSION

Theoretically, HIV-1 may remain latent in a nonreplicating provirus for undetermined periods of time before the development of HIV antibodies. Infected individuals may harbor HIV-1 detectable by culture or PCR for months to years after exposure to the virus before seroconversion as defined by commercially available antibody assays.7,9,10,13 PCR has the unique ability to detect minute quantities of HIV-1 specific nucleic acid independently of viral replication or host immune response.

Several investigators have examined different groups of high-risk seronegative subjects for evidence of HIV-1 DNA by PCR. In 1989, Imagawa et al.7 first reported that HIV-1 infection in some homosexual men at high risk could be detected at least 35 months before seroconversion. Subsequently, Wolinsky et al.8 found that HIV-1 infection could be detected by PCR 6 to 42 months before a diagnostic Western blot assay in 20 of 24 homosexual men who continued to engage in high-risk behavior. Hewlett et al.9 reported that 11 (50%) of 22 seronegative female sexual partners of HIV-1 infected hemophiliacs were PCR positive. These studies suggest that there may be a prolonged seronegative state after acquisition of HIV-1 infection in some individuals.

However, Horsburgh et al.10 found that infection for longer than 6 months without detectable antibody was uncommon in 27 homosexual and 12 hemophiliac men for whom samples were available before and after seroconversion. Jackson et al.11 found none of 20 seronegative multitransfused hemophiliacs to be PCR positive, and Sullivan et al. found no silent infections among 20 seronegative female spouses with more than 5 years sexual exposure to an HIV-infected hemophiliac male (reference 15 and personal communication, J.L. Sullivan, August 1990). Mariotti et al.12 found no HIV-1 proviral DNA in 76 high-risk seronegative individuals, including 20 polytransfused hemophiliacs. These findings concur with our data, which suggest that the rate of HIV infection among high-risk antibody negative individuals with hemophilia is very low to absent, in the range of 0% to 2%.

These conflicting results must be interpreted with caution, because PCR, although capable of detecting extremely small quantities of HIV-1 genetic material (as few as 1 infected in 100,000 uninfected cells), is fraught with technical problems. Many investigators have experienced problems with false positive PCR results due to laboratory contamination. To decrease the likelihood of carryover, we paid strict attention to separation of pre- and postamplification processing, and included multiple negative and positive patient samples as internal controls with each set of assays. Positive results were confirmed using separate blinded aliquots of cell lysates from the same date.

The low frequency of PCR positive, seronegative hemophiliacs in our cohort could be due to lower sensitivity of our PCR methodology. However, two things argue against lack
of sensitivity having a significant impact on these data. First, we were consistently able to detect all the HIV-1 seropositive clinical specimens processed in a blinded manner in parallel with our negative specimens. Second, we have not had any seroconversions during the past 5 years in our study population, in contrast with the continuing seroconversions observed in the Multicenter AIDS Cohort (MAC) study.

HIV-1 proviral DNA was demonstrated in two separate aliquots of PBMC from a single subject. However, a follow-up specimen on this child was negative. Others have noted that HIV-1 amplified products were not detected consistently at all time points during long latency periods preceding seroconversion. Either sampling error or episodic HIV replication in cells eventually cleared from the circulation could account for these results. Simmonds et al have reported that PBMC DNA from one seropositive individual contained only five copies of provirus per $10^6$ cells. With only 1 positive test in 57, the Poisson model would predict that nearly 1 million cells would be required to insure a 90% probability of obtaining a positive test result at this level of detection. Thus, considerably more than 1 $\mu$g of DNA from 150,000 cells would be needed to insure reproducibly positive PCR results. Alternatively, the single positive result in this individual who remains seronegative and culture negative more than 2 years since his most recent exposure to nonheat-treated products could have been due to sample contamination. Continued follow-up will be required to determine whether this isolated finding represents a false negative due to decreased sensitivity or a false positive due to lack of specificity.

The observation that only 1 of 57 (1.8%) seronegative recipients repeatedly exposed to HIV-1 contaminated blood products more than 3 years previously had possible evidence of PCR reactivity in a cross-sectional cohort study indicates that silent or controlled infections in multitransfused HIV-1 negative hemophiliacs are infrequent, if they exist. These findings should provide considerable reassurance to seronegative persons with hemophilia and their sexual partners.

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REFERENCES

Silent infections with human immunodeficiency virus type 1 are highly unlikely in multitransfused seronegative hemophiliacs [see comments]

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