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Human Immunodeficiency Virus Type 1 Detected in All Seropositive Symptomatic and Asymptomatic Individuals

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Between February 1987 and October 1988, peripheral mononuclear blood cells (PBMC) from 409 adult individuals antibody positive by Western (immuno-) blot for human immunodeficiency virus type 1 (HIV-1) (56 acquired immunodeficiency syndrome [AIDS] patients, 88 patients with AIDS-related complex, and 265 asymptomatic individuals) were consecutively cultured for HIV-1 or tested for the presence of HIV-1 DNA sequences by a polymerase chain reaction assay (PCR). We isolated HIV-1 or detected HIV-1 DNA sequences from the PBMC of all 409 HIV-1 antibody-positive individuals. None of 131 healthy HIV-1 antibody-negative individuals were HIV-1 culture positive, nor were HIV-1 DNA sequences detected by PCR in the blood specimens of 43 seronegative individuals. In addition, HIV-1 PCR and HIV-1 culture were compared in testing the PBMC of 59 HIV-1 antibody-positive and 20 HIV-1 antibody-negative hemophiliacs. Both methods were found to have sensitivities and specificities of at least 97 and 100%, respectively. In contrast, the sensitivities of serum HIV-1 antigen testing in AIDS patients and asymptomatic seropositive patients were 42 and 17%, respectively. Our ability to directly demonstrate HIV-1 infection in all HIV-1 antibody-positive individuals provides definitive support that HIV-1 antibody positivity is associated with present HIV-1 infection. Moreover, the sensitivities and specificities of PCR and culture for the detection of HIV-1 appear to be equivalent, and both methods are superior to testing for HIV-1 antigen in serum for the direct detection of HIV-1.

The role of human immunodeficiency virus type 1 (HIV-1) as the cause of the acquired immunodeficiency syndrome (AIDS) has been challenged (4, 5) because HIV-1 was not isolated from 6 to 50% of HIV-1-seropositive AIDS cases reported (3, 7, 17, 18). The culture recovery rate of HIV-1 from HIV-1 antibody-positive asymptomatic patients has generally been even lower, only 20 to 42% in some studies (1, 6, 18). However, at least three studies have reported 100% isolation rates of HIV-1 from AIDS patients studied (1, 6, 9), but we are aware of no study in which HIV-1 has been isolated or directly detected in hundreds of consecutively tested HIV-1-seropositive symptomatic and asymptomatic patients. In this report, we demonstrate the presence of HIV-1 infection in all 409 consecutive HIV-1 antibody-positive patients (including 144 patients with AIDS or AIDS-related complex [ARC]) tested by a virus isolation procedure or a polymerase chain reaction (PCR) technique for the detection of HIV-1 DNA sequences. Moreover, we demonstrate that PCR analysis is as sensitive and specific as current culture techniques for the detection of HIV-1.

MATERIALS AND METHODS

Between February 1987 and October 1988, peripheral blood mononuclear cells (PBMC) from 409 individuals who were antibody positive for HIV-1 by Western (immuno-) blot (56 AIDS patients, 88 patients with ARC, and 265 asymptomatic individuals) were consecutively cultured for HIV-1 by using a sensitive technique previously described in several earlier reports involving 199 of these subjects (9–11). All subjects tested represented different individuals whose serum antibody test results were Western blot positive for HIV-1 and had not been previously cultured for HIV-1. All 409 subjects were adult homosexual males or male intravenous drug abusers, with the exception of 59 male hemophilia patients and 8 women. The mode of HIV-1 transmission for the eight women was as follows: heterosexual sex, four; intravenous drug use, two; transfusion, two. At the time of venipuncture, none of the patients were known to be taking drugs that had anti-HIV-1 activity. Blood samples from 131 healthy HIV-1 antibody-negative individuals, including 20 seronegative hemophiliacs, were also cultured for HIV-1. Briefly, PBMC were separated from 30 ml of heparinized blood on a Ficoll-Paque (Pharmacia, Inc., Piscataway, N.J.) gradient, and 1×10^7 cells were placed in a 50-ml tissue culture flask containing 15 ml of RPMI 1640 medium–20% fetal calf serum–5% interleukin-2–Polybrene (Sigma Chemical Co., St. Louis, Mo.) (5 μg/ml)–penicillin (200 U/ml)–streptomycin (200 μg/ml) and cocultured with 5×10^6 phytohemagglutinin-stimulated PBMC from an HIV-1 antibody-negative blood donor. Cocultures were incubated at 37°C in a 5% CO_2 atmosphere for up to 42 days. Approximately 8 ml of culture medium above the settled PBMC was removed twice weekly for testing and replaced with an equal volume of fresh medium. An additional 3×10^6 phytohemagglutinin-stimulated donor PBMC were added weekly. Culture supernatant fluids removed twice a week for as long as 6 weeks were stored as 1-ml samples at −20°C.

After thawing, supernatants were tested for HIV-1 antigen

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by using a commercially available enzyme immunoassay that detects primarily the core p24 antigen of HIV-1 (8) (Abbott Laboratories, North Chicago, Ill.). A culture was considered positive if two serial supernatant fluid samplings were positive, with the later sampling demonstrating at least a twofold increase in reactivity. Culture supernatant fluids from known HIV-1-positive and HIV-1-negative cell cultures were included in every assay as positive and negative controls.

In addition, serum samples from 403 of the 409 HIV-1 antibody-positive individuals were tested for HIV-1 antigen with the same enzyme immunoassay used for detection of HIV-1 in culture supernatant fluids (8). The lower limit of detectability for this assay is approximately 50 pg/ml (14). Reactive specimens were retested by using a neutralization assay for specificity (14).

To confirm the presence of HIV-1 infection in the antibody-positive, culture-negative subjects, a rapid lysis-extraction technique and a PCR assay were used to detect HIV-1 *gag* sequences (10). Briefly, DNA was extracted from frozen uncultured PBMC collected at the time of initial testing by the following technique. Cells were rapidly thawed, transferred to 2-ml Sarstedt tubes, and pelleted by centrifugation for 10 min at 3,000 rpm in a variable-speed Microfuge. The pellets were washed once with 2 ml of RPMI 1640 medium containing 10% fetal calf serum and twice with 2 ml of phosphate-buffered saline. The cell pellets were suspended to a concentration of approximately 2×10^6 cells per ml in a solution containing 100 mM KCl, 10 mM Tris hydrochloride (pH 8.3), and 2.5 ml of $MgCl_2$. Following suspension, an equal volume of a solution containing 10 mM Tris hydrochloride (pH 8.3), 2.5 mM $MgCl_2$, 1% Tween 20, 1% Nonidet P-40, and 120 μg of proteinase K per ml was added. The samples were digested with proteinase K for 1 h at 60°C, after which the proteinase K was inactivated at 95°C for 10 min.

DNA from the equivalent of 150,000 to 1,500,000 cells (1 to 10 μg) was analyzed by PCR first with a primer pair that amplifies a conserved region of HLA-DQa to determine whether the DNA in the crude cell lysates was of sufficient quality for amplification. All amplifications were performed with the *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus Inc., Norwalk, Conn.) for 30 cycles on an automated thermal cycler (Perkin-Elmer Cetus), as described previously in detail (16). Each cycle started with a 25-s denaturation at 95°C, followed by cooling and holding at 55°C for 25 s to allow for primer annealing. Each cycle ended with a chain elongation step of 60 s at 72°C.

Amplifications of HIV-1 were performed by using a primer pair, SK38-39 (13), which amplifies a 115-base-pair conserved region of the *gag* gene (nucleotides 1551 to 1665 of HIV SF2; GenBank accession no. K02007). The amplified product was detected by oligomer hybridization, a technique in which a ^{32}P-end-labeled probe (SK19) to the nucleotide 1595 to 1635 *gag* region hybridizes in solution to one strand of the amplified sequence. The probe-target duplex was then resolved by electrophoresis on a 10% polyacrylamide gel and autoradiographed. DNA samples from the PBMC of 23 healthy heterosexual HIV-1 antibody-negative, culture-negative individuals were also tested by PCR assay for the presence of HIV-1 *gag* sequences.

In addition, the PBMC of 59 seropositive and 20 seronegative hemophiliacs were analyzed by PCR for HIV-1 *gag* sequences in order to compare the sensitivities and specificities with those of HIV-1 culture. A total of 44 of the 59 (75%) seropositive hemophiliacs and all 20 seronegative hemophiliacs were asymptomatic for HIV-1. The technologist who performed the PCR analysis and cultures was blinded as to the HIV-1 antibody status of the hemophiliacs tested.

Statistical analyses were performed with the Wilcoxon rank sum test or the chi-square test with Yates correction, as appropriate. All *P* values are reported as two-tailed.

TABLE 1. Cumulative proportion of positive HIV-1 cultures from antibody-positive patients by day in coculture

No. of days in culture	Cumulative no. (%) of positive cultures for patient group		
	AIDS (n = 56)	ARC (n = 88)	Asymptomatic (n = 265)
3	30 (54)	28 (32)	36 (14)
7	53 (95)	66 (75)	190 (68)
10	54 (97)	77 (88)	217 (82)
14	55 (98)	81 (92)	235 (89)
17	56 (100)	81 (92)	244 (92)
21		84 (95)	250 (94)
24		85 (97)	254 (96)
28		86 (98)	255 (96)
35		87 (99)	258 (97)
38			259 (98)

RESULTS

Initially, HIV-1 was cultured from the blood specimens of 398 of 409 (97.3%) HIV-1 antibody-positive individuals, including all 56 AIDS patients. Subsequent blood specimens for a repeat culture were obtained from 7 of the 11 culture-negative subjects 4 to 24 weeks later. HIV-1 was detected in 4 of these 7 individuals, for an overall virus detection rate of 98.3% (402 of 409) that included 143 of 144 (99.3%) HIV-1-symptomatic patients (Table 1). None of the 131 HIV-1 antibody-negative individuals had positive cultures. The median time to positive culture for AIDS patients was significantly shorter compared with that for asymptomatic patients (<3.0 versus 4 to 7 days; $P < 0.001$, Wilcoxon rank sum test). The proportion of HIV-1 antibody-positive AIDS patients in whom HIV-1 antigen was directly detected in serum samples was significantly greater compared with that of asymptomatic individuals (42 versus 17%, $P < 0.001$; Table 2).

All initial DNA samples from the 7 HIV-1 antibody-positive, culture-negative patients generated the 115-base diagnostic HIV-1 fragment (Fig. 1), whereas this HIV-1 sequence was not detected by any DNA samples from 43 HIV-1 antibody-negative, culture-negative individuals.

The results of the HIV-1 PCR assay and HIV-1 culture comparison in testing hemophiliacs revealed the following. A total of 57 of 59 (97%) of HIV-1 antibody-positive hemo-

TABLE 2. Proportion of HIV-1 antibody-positive patients with detectable HIV-1 antigen in serum

Patient group	No. with antigen/total (%)
AIDS	23/56 (42)[a]
ARC	31/88 (37)
Asymptomatic	44/259 (17)[a]
Total	98/403 (24)

[a] $P < 0.001$ for data for AIDS patients and asymptomatic patients. Corrected chi-square (χ^2) = 14.6.

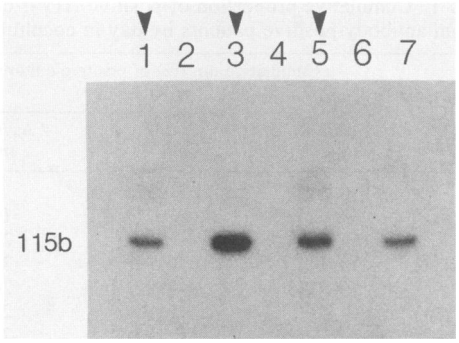

FIG. 1. Representative DNA amplification analysis of PBMC DNA from three HIV-1 antibody-positive, culture-negative individuals. Autoradiograph shows amplified 115-base HIV-1 *gag* sequence after 30 cycles of amplification. Lanes: 1, 3, and 5, DNA from HIV-1 antibody-positive, culture-negative individuals; 2 and 4, DNA from HIV-1 antibody-negative, culture-negative individuals; 6, 1 μg of human placental DNA; 7, 25 pg of human DNA from a cell line established from an HIV-1-infected individual.

philiacs were positive by culture, and 57 of 59 (97%) were positive by PCR analysis. However, the two culture-negative, antibody-positive asymptomatic hemophiliacs were PCR positive, and the two PCR-negative, antibody-positive asymptomatic subjects were culture positive, so that all seropositive hemophiliacs (59 of 59) were either culture positive and/or PCR positive. No (0 of 20) seronegative hemophiliacs were culture or PCR positive. We repeated the PCR analysis on the same DNA samples from the two culture-positive, PCR-negative individuals, and one individual was clearly positive the second time, suggesting that the sensitivity of PCR with one primer pair may exceed 98% if samples are run in duplicate. The use of two or more HIV-1 primer pairs could further increase this sensitivity (13). The median time to confirm culture positivity was 10 days, with a range of 10 to 28 days.

None of the HIV-1 antibody-negative hemophiliacs were positive by PCR or culture.

DISCUSSION

Our results indicate that HIV-1 can be isolated from 100% (56 of 56) of AIDS patients, 99% (87 of 88) of ARC patients, and 98% (259 of 265) of HIV-1 antibody-positive asymptomatic individuals. In contrast, the sensitivities of serum antigen testing for HIV-1 in these same patient groups were only 42, 37, and 17%, respectively. When PCR was used to supplement virus isolation, direct evidence of HIV-1 infection was detected in all 409 HIV-1 antibody-positive individuals tested. While this finding is not evidence of active viral replication, it does confirm that AIDS patients as well as HIV-1 antibody-positive asymptomatic individuals harbor HIV-1 genetic material. The shorter time to positive culture for AIDS patients compared with that for asymptomatic individuals suggests that AIDS patients have a greater viral load. The report that each 100-fold dilution of HIV-1 stock virus increases the time to positive culture by approximately 4 to 7 days supports this suggestion (15). The conclusion that AIDS patients have a greater viral antigenic load than asymptomatic individuals is further supported by the significantly greater percentage of AIDS patients with detectable HIV-1 antigenemia compared with asymptomatic individuals (Table 2). These data strongly support the results of smaller studies in which symptomatic HIV-1 infection was associated with a greater HIV-1 viral load and/or active HIV-1 replication (2, 6, 8, 11). For example, Ehrnst et al. have recently shown that when plasma was sufficiently concentrated, HIV-1 could be isolated from 9 of 9 (100%) AIDS patients but from only 7 of 13 (54%) seropositive asymptomatic individuals (6). Another explanation for shorter time to positivity in AIDS patients is that they have more-virulent strains of HIV-1 that replicate more efficiently in vitro.

Comparison of HIV-1 culture and PCR analysis showed similar sensitivities (97%) and excellent specificities (100%) in the detection of HIV-1 infection. However, PCR analysis has the advantages of faster turnaround time, smaller sample requirements, and noninfectious viral amplification. The addition of a second or third primer pair to highly conserved regions of the HIV-1 genome could further increase the sensitivity of PCR analysis in case of nucleotide variation in one region of a particular HIV-1 strain resulting in a false-negative result. On the other hand, the exquisite sensitivity of PCR makes it essential to prevent and detect contamination of samples by previously amplified sequences in the laboratory by meticulous attention to procedural details, the use of appropriate controls, and consistency of results (12). It should also be noted that PCR analysis as done in this study does not distinguish active from latent infection or defective from nondefective virus.

In summary, we were able to definitively demonstrate that HIV-1 infection is present in all HIV-1 antibody-positive adults, regardless of clinical status, by using a sensitive culture or PCR assay for detection of the virus. Certainly the argument that HIV-1 is not the cause of AIDS because it is not present in all HIV-1-seropositive AIDS patients is no longer tenable. This large study confirms earlier reports that all HIV-1 antibody-positive adults are at risk for the development of AIDS. In addition, recently licensed serum antigen tests for HIV-1 appear to have limited utility in the direct detection of HIV-1 in antibody-positive patients.

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