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Detection of Human Immunodeficiency Virus Type 1 (HIV-1) Antibody by Western Blotting and HIV-1 DNA by PCR in Patients with AIDS

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The human immunodeficiency virus type 1 (HIV-1) Western blotting (immunoblotting) band patterns and the sensitivity of an HIV-1 DNA PCR assay were determined by testing the blood of patients with AIDS. Plasma and cell pellets processed from the peripheral blood of 199 patients with absolute CD4 cell counts of less than 200 cells per mm^3 were tested by a licensed enzyme immunoassay (EIA; Abbott HIV-1) and Western blot assay (Cambridge-Biotech) for HIV-1 antibody. The Roche HIV-1 AMPLICOR DNA PCR assay was used to test cell pellets from 125 of the 199 patients for HIV-1 *gag* DNA sequences. All plasma samples from these 199 sequential patients were reactive for HIV-1 antibody by EIA and were positive by Western blot assay using the criteria recommended by the Centers for Disease Control and Prevention. The majority of samples (192 of 199; 96.5%) displayed at least six of nine bands characteristic of the virus by Western blotting, with the lowest number of bands characteristic of the virus displayed by any sample being three. However, 39 and 48% of all patients exhibited no bands to p17 and p55 antigens, respectively, whereas 0 to 7.5% of all patients exhibited no bands to the other antigens. HIV-1 *gag* DNA sequences were detected in 117 (93.6%) of 125 cell pellets processed from the peripheral blood of these same patients. All eight patients initially negative by PCR tested positive when a second pellet which had been produced from the same blood sample was tested. Despite a decrease in antibody reactivity to HIV Gag and Pol proteins, patients with advanced HIV-1 infection remained positive for HIV-1 antibody by EIA and Western blot testing. Confirmation by the HIV-1 Western blot assay still appears to be the more sensitive assay for the diagnosis of HIV-1 infection in those individuals with advanced HIV-1 infection in the United States.

Licensed enzyme immunoassay (EIA) kits are most commonly used to test the blood of individuals for antibody to human immunodeficiency virus type 1 (HIV-1) (7). If the patient sample is repeatedly reactive by EIA, then a licensed Western blot (immunoblot) assay is most often performed to verify the presence of HIV-1 antibody (5, 7, 9, 10, 22). This assay typically reveals up to nine bands characteristic of the virus if antibodies to HIV-1 proteins of different molecular weights are present (6, 7, 9, 10).

Loss of antibody to HIV-1 has been reported to occur in patients with advanced HIV-1 infection, presumably due to progressive impairment of humoral immunity (1–3, 12, 17–20). In particular, these studies have noted complete or partial loss of antibody reactivity to the HIV-1 Gag proteins in patients with AIDS (1, 2, 13, 16–21). Since antibody reactivity to two of three antigens (p24, gp41, and gp120/160) is necessary for a diagnosis of HIV infection by Western blotting according to criteria recommended by the Centers for Disease Control and Prevention (CDC) (6), loss of antibody reactivity to two or more of these antigens could result in an indeterminate or negative Western blot assay result, thereby jeopardizing the ability of antibody assays to reliably diagnose HIV-1 infection in patients presenting with advanced infection.

Studies conducted in the 1980s showed that the Western blot assay has excellent sensitivity in confirming HIV-1 infection by using the CDC criteria (7, 9). However, since the 1980s, patients with advanced disease have been surviving longer, and the virus has most likely continued to evolve and diversify. It is possible that some patients with advanced HIV-1 infection lack antibodies to specific HIV-1 proteins, such that the results of currently licensed Western blot assays are indeterminate or even negative when the CDC interpretative criteria are used. At the same time, it has been suggested that detection of conserved HIV-1 DNA sequences by PCR technology may be more sensitive for the diagnosis of HIV-1 infection (15). Therefore, we undertook this study in 1994 (i) to determine the Western blot assay band patterns for a large number of HIV-1-infected patients with AIDS and (ii) to determine the sensitivity of the Roche HIV-1 AMPLICOR DNA PCR assay in detecting HIV-1 DNA in the peripheral blood of this same population.

MATERIALS AND METHODS

Specimen processing. Peripheral blood samples from 199 different individuals with absolute CD4 cell counts of less than 200 cells per mm^3 were collected in EDTA anticoagulant. The samples were obtained sequentially from the Clinical Immunology Laboratory at the University Hospitals of Cleveland (Cleveland, Ohio), where absolute CD4 cell counts in the blood of patients attending the HIV clinic had been determined earlier in the day. Patients were on a variety of antiretroviral therapies and drugs to prevent or treat opportunistic infections. Patients also varied in their stage of clinical disease, from asymptomatic to critically ill. However, all patients had AIDS according to the CDC definition because all patients had CD4 cell counts of less than 200 cells per mm^3 (8). Samples were unlinked with patient identifiers and then separated into plasma and frozen within 24 h of collection for HIV antibody testing. Cell pellets obtained from the last 125 of the 199 blood samples collected were also processed for PCR testing for HIV-1 DNA. In brief, two to four 0.5-ml aliquots of

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TABLE 1. Percentage of patient sera showing various numbers of bands by Cambridge Biotech Western blot assay[a]

No. of bands	No. of patients	% of patients	Sum (%)
9	79	39.7	39.7
8	61	30.7	70.4
7	43	21.6	92.0
6	9	4.5	96.5
5	3	1.5	98.0
4	1	0.5	98.5
3	3	1.5	100
2	0	0.0	100
1	0	0.0	100

[a] Sera from 199 patients were tested.

whole blood were each processed into cell pellets within 24 h of collection for PCR testing for HIV-1 DNA according to the directions of the manufacturer of the HIV-1 AMPLICOR DNA PCR assay kit (Roche Molecular Systems, Branchburg, N.J.). Each cell pellet consisted of the total number of leukocytes contained in 500 μl of whole blood after erythrocyte lysis with whole blood specimen wash solution. The cell pellets were frozen at −70°C until amplification and detection, which were performed within 1 month of collection. Plasma was separated from the remaining whole blood and was frozen at −70°C for HIV-1 antibody testing.

In addition, identical testing was performed with specimens from 21 volunteer blood donors who tested negative by a licensed EIA, the HIV-1/2 antibody kit (Abbott Laboratories, Abbott Park, Ill.), at the time of donation in 1993.

HIV-1 antibody testing. All plasma samples were tested by a licensed HIV-1 EIA (Abbott Laboratories) and a licensed HIV-1 Western blot kit (Cambridge Biotech Corporation, Worcester, Mass.). The assays were performed and the results were interpreted according to the manufacturers' directions, with the exception that the EIA was not repeated if the initial absorbance reading was >2.0. A Western blot assay result was considered negative if no bands were present and positive if at least two of the following bands were present: p24, gp41, and gp120/160 (6). A Western blot assay result that did not meet the criteria for positive or negative was considered indeterminate. The nine bands (according to molecular weight) considered to be characteristic of HIV-1 on Western blots were as follows: p17, p24, p31, gp41, p51, p55, p66, gp120, and gp160. The intensity of band reactivity was scored as described in the package insert, as follows: 0, absent; 0.5, less than the intensity of the p24 band on the weakly reactive control strip; 1.0, at least as intense as the p24 band on the weakly reactive control strip but less intense than the p24 band on the strongly reactive control strip; 2.0, greater than or equal to the intensity of the p24 band on the strongly reactive control strip.

PCR testing for HIV-1 DNA. Cell pellets prepared from whole blood from each donor were incubated with 200 μl of extraction reagent at 60°C for 30 min and then at 98°C for 30 min. Fifty microliters of cell lysate, equivalent to an estimated 125,000 to 600,000 peripheral blood mononuclear cells, was added to 50 μl of a master mixture containing dATP, dCTP, dGTP, and dUTP (dUTP was used instead of dTTP), AmpErase, *Taq* polymerase, and biotinylated SK462 and SK431 *gag* primers. Amplification was performed in duplicate on a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.) by using the following cycling parameters: 50°C for 2 min; 5 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; and 30 cycles of 90°C for 10 s, 60°C for 10 s, and 72°C for 10 s. A total of 100 μl of the amplified, biotinylated 142-bp product was then denatured with 100 μl of denaturation solution for 10 min at room temperature. A 25-μl sample of denatured product was then added to 100 μl of hybridization solution in a microwell coated with the capture probe (SK102) for 1 h at 37°C. The microwells were then washed, and 100 μl of avidin-horseradish peroxidase conjugate was added to each well and the plate was incubated for 15 min at 37°C. After a final wash, a tetramethylbenzidine-H_2O_2 solution was added to each well and the plate was incubated for 10 min at 23 to 24°C. One hundred microliters of stop reagent was added to each well to stop the reaction. The absorbance of each well at 450 nm was determined within 1 h by using a microwell plate reader. A result was judged to be positive if the optical density of the sample tested was greater than 0.35. If the results were discrepant (i.e., positive or negative according to the reader), then a second pellet was extracted, 50 μl of cell lysate was amplified in duplicate, and 25 μl of denatured product was detected in singleton to resolve the discrepancy. Both amplifications had to have tested either positive or negative for resolution of the results; otherwise, the result was considered to be indeterminate.

In order to monitor the sensitivity and specificity of the assay, known positive and negative controls provided with the kit were run with each assay. The positive control consisted of a 20-copy plasmid containing a noninfectious HIV-1 genome from the 8E5 cell line (11). For a run to be valid, the negative control reagent had to test negative and the positive control had to test positive.

PCR testing for HIV-1 DNA was performed by two medical technologists, each of whom had several years of experience with the Roche HIV-1 AMPLICOR DNA PCR assay in our AIDS Clinical Trials Group Virology Laboratory and had successfully completed testing of Roche HIV-1 validation panels.

Statistical analysis. Data were analyzed by standard methods for determining means, medians, and standard deviations. Wilcoxon ranked sum scores were used to analyze differences in CD4 cell counts between samples with low and high antibody band intensities. SAS for Windows, version 6.08, was used for all statistical calculations.

RESULTS

HIV-1 antibody testing. One hundred ninety-nine (100%) of 199 plasma samples from sequential patients with absolute CD4 cell counts of less than 200 cells per mm^3 were reactive for HIV-1 antibody by EIA, with all samples exhibiting absorbances of >2.0. All 199 samples were positive by Western blotting by using the CDC criteria. The mean and median CD4 cell counts for this group were 57 and 40 cells per mm^3, respectively (range, 0 to 190 cells per mm^3). In this study, 39.7% of plasma samples from patients with AIDS exhibited all nine bands characteristic of the virus on Western blotting, 96.5% of patients exhibited at least six of nine characteristic bands, and all patients exhibited at least three characteristic bands, which was sufficient to meet the CDC criteria for a positive Western blot assay result (Table 1).

However, 38.7 and 48.2% of all patients exhibited no bands to the p17 and p55 Gag antigens, respectively, whereas 0 to 7.5% of all patients exhibited no bands to the other antigens (Table 2). Likewise, the intensities of the band reactivities to both Gag and Pol proteins (p17, p24, p31, p51, p55, and p66) were less for patients with lower CD4 cell counts (Table 3). However, this association was only statistically significant for reactivity to the p66 protein ($P = 0.019$).

PCR testing for HIV-1 DNA. HIV-1 *gag* DNA sequences were detected in cell pellets of sera from 117 (93.6%) of 125

TABLE 2. Frequency of virus-specific bands on Western blot assays for sera from patients with AIDS[a]

Band	No. of patients with the following no. of bands present on Western blotting/total no. of patients (%)									Total
	9 bands	8 bands	7 bands	6 bands	5 bands	4 bands	3 bands	2 bands	1 band	
p17	79/79 (100)	40/61 (65.6)	2/43 (4.7)	0/9 (0)	1/3 (33.3)	0/1 (0)	0/3 (0)	0/0 (0)	0/0 (0)	122/199 (61.3)
p24	79/79 (100)	60/61 (98.4)	40/43 (93)	3/9 (33.3)	1/3 (33.3)	0/1 (0)	1/3 (33.3)	0/0 (0)	0/0 (0)	184/199 (92.5)
p31	79/79 (100)	61/61 (100)	43/43 (100)	6/9 (66.7)	1/3 (33.3)	0/1 (0)	0/3 (0)	0/0 (0)	0/0 (0)	190/199 (95.5)
gp41	79/79 (100)	61/61 (100)	43/43 (100)	9/9 (100)	3/3 (100)	1/1 (100)	3/3 (100)	0/0 (0)	0/0 (0)	199/199 (100)
p51	79/79 (100)	61/61 (100)	43/43 (100)	9/9 (100)	0/3 (0)	1/1 (100)	0/3 (0)	0/0 (0)	0/0 (0)	193/199 (97)
p55	79/79 (100)	22/61 (36.1)	1/43 (2.3)	0/9 (0)	1/3 (33.3)	0/1 (0)	0/3 (0)	0/0 (0)	0/0 (0)	103/199 (51.8)
p66	79/79 (100)	61/61 (100)	43/43 (100)	9/9 (100)	2/3 (66.7)	1/1 (100)	0/3 (0)	0/0 (0)	0/0 (0)	195/199 (98)
gp120	79/79 (100)	61/61 (100)	43/43 (100)	9/9 (100)	3/3 (100)	0/1 (0)	2/3 (66.7)	0/0 (0)	0/0 (0)	197/199 (99)
gp160	79/79 (100)	61/61 (100)	43/43 (100)	9/9 (100)	3/3 (100)	1/1 (100)	3/3 (100)	0/0 (0)	0/0 (0)	199/199 (100)

[a] Sera from 199 patients were tested.

TABLE 3. Association of mean CD4 cell count with intensity of band reactivity[a]

Band	Mean ± 1 SD CD4 cell count/mm^3 (no. of serum samples)		*P* value
	Intensity score, ≤0.5	Intensity score, ≥1.0	
p17	54 ± 54 (100)	59 ± 58 (99)	0.528
p24	36 ± 36 (28)	60 ± 58 (171)	0.069
p31	44 ± 53 (19)	58 ± 57 (180)	0.201
gp41	0.0 (0)	57 ± 56 (199)	
p51	34 ± 38 (15)	59 ± 57 (184)	0.124
p55	52 ± 53 (127)	64 ± 62 (72)	0.192
p66	23 ± 29 (12)[b]	59 ± 57 (187)[b]	0.019[b]
gp120	67 ± 15 (3)	57 ± 57 (196)	0.3405
gp160	0.0 (0)	57 ± 57 (199)	

[a] Sera from 199 patients were tested.
[b] $P < 0.05$.

patients tested. All eight patients initially negative by PCR tested positive when a second pellet which had been produced from the same blood sample was tested.

All 21 volunteer blood donors tested negative for HIV-1 antibody by EIA and Western blot assay and had no detectable HIV-1 *gag* DNA sequences by the Roche HIV-1 AMPLICOR DNA PCR assay.

DISCUSSION

Our results confirm that HIV-1 antibody testing by EIA followed by Western blotting is a very sensitive algorithm for detecting HIV-1 in patients with advanced HIV-1 infection. The percentage of Western blots exhibiting a large number of bands characteristic of the virus was, in fact, higher than that reported in the Cambridge Biotech package insert for 52 patients with AIDS for any given band (4).

Less intense band reactivities to all Gag and Pol antigens on Western blotting were seen with plasma from patients with lower CD4 cell counts, although the difference was only significant for reactivity to p66. However, we believe if differences in band intensity had been studied in patients with a wider range of CD4 cell counts, the differences would have been greater, as has been reported elsewhere (9). In contrast, there was no difference in band intensity for the envelope bands (gp41 and gp120/160) for patients whose CD4 cell counts varied from 0 to 200 cells per mm^3. Although a decrease or loss of reactivity to HIV Gag and Pol proteins appears to occur in patients with lower CD4 cell counts, sufficient numbers and types of bands are still present for a diagnosis of HIV-1 infection.

One could argue that since the presence of envelope bands on Western blots is required for the diagnosis of HIV-1 infection and since we have selected only those patients who had a diagnosis of HIV-1 infection, then it is not surprising that all of the patients in this study had at least one envelope band. However, patients were not selected for this study on the basis of a current Western blot assay result but on the basis of their CD4 cell counts and a previous diagnosis of HIV infection. For many of these patients, a Western blot assay had not been performed for years. Therefore, there was an opportunity for changes in antibody reactivity to occur during the course of HIV-1 infection.

In this study, the HIV-1 Western blot assay appeared to be a more sensitive assay than the prototype Roche HIV-1 AMPLICOR DNA PCR assay for confirming HIV-1 infection in patients with advanced infection. Testing of patient peripheral blood leukocytes by the Roche HIV-1 AMPLICOR DNA PCR assay revealed that 93.6% of patients had detectable HIV-1 *gag* DNA sequences. This percentage is consistent with the results reported in an earlier study (14), but it is less than what we expected in testing patients with advanced HIV-1 infection by technologists proficient with the assay. The fact that HIV-1 *gag* DNA sequences were able to be detected on repeat testing suggests that a different viral subtype is unlikely to explain the false-negative results. It appears more likely that the false-negative results were due to technical errors in either processing the specimens or performing the assay.

In summary, despite a loss of antibody reactivity to HIV-1 Gag and Pol proteins, most, if not all, patients with advanced HIV-1 infection remain positive for HIV-1 antibody by EIA and Western blot assay in the midwestern United States. In contrast, 6.4% of patients with advanced HIV infection initially tested negative by the Roche HIV-1 AMPLICOR DNA PCR assay. The HIV-1 Western blot assay still appears to be the more sensitive assay for confirmation of the diagnosis of HIV-1 infection in those individuals with advanced HIV-1 infection in the United States. With the introduction of different HIV-1 subtypes in the United States, it will be important to monitor the ability of current EIA and Western blot assays to reliably detect HIV infection (13).

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