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Evaluation of Infection with Human Immunodeficiency Virus Type 1 by Using Nonisotopic Solution Hybridization for Detection of Polymerase Chain Reaction-Amplified Proviral DNA

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A convenient assay combining solution hybridization and enzyme immunoassay for DNA-RNA hybrids (polymerase chain reaction-enzyme immunoassay [PCR-EIA]) was developed to detect human immunodeficiency virus type 1 (HIV-1) provirus amplified by the PCR and was compared with oligomer hybridization with ^{32}P-labeled SK19. In PCR-EIA, a fragment of the HIV-1 *gag* gene from peripheral blood mononuclear cells was first amplified with primer pair SK38/SK39 or O1/O2. PCR-amplified material was reacted in solution with a biotinylated RNA probe. Biotinylated hybrids were measured in a microtiter-plate EIA with antibiotin antibody and a β-D-galactosidase-conjugated monoclonal antibody to DNA-RNA hybrids. Ten copies of HIV-1 DNA could be detected by PCR-EIA by using two different sets of primers. HIV-1 DNA was detected in 104 of 108 peripheral blood mononuclear cell samples by using SK38/39 and oligomer hybridization, in 104 of 108 samples by using SK38/SK39 and PCR-EIA, and in 104 of 108 samples by using O1/O2 and PCR-EIA. HIV-1 provirus was detected in 107 of 108 samples by using a combination of two sets of primers. One sample from a seropositive patient was negative in all three PCR assays, and six samples gave discordant results between primer pairs. Six of the latter samples scored negative in a PCR for β-globin but became positive when the sample was diluted before amplification. When applied to clinical samples, PCR-EIA generated results similar to those of an isotopic assay for detection of amplified DNA.

The diagnosis of human immunodeficiency virus type 1 (HIV-1) infection relies mainly on the detection of specific antibodies directed against viral proteins (16). Direct detection of HIV-1 in biological fluids would be useful in selected circumstances but has been hampered by the hazardous and lengthy procedures required for HIV-1 culture (9). Neither antigen detection nor hybridization assays that directly identify the presence of HIV-1 sequences in samples have reached satisfactory levels of sensitivity (16). To overcome the poor sensitivity of hybridization assays, an in vitro amplification method, the polymerase chain reaction (PCR), has been applied for detection of HIV-1 in peripheral blood mononuclear cells (PBMC) (28).

PCR selectively amplifies up to a millionfold specific DNA sequences and allows for the detection of minute amounts of DNA (32). The synthesis of short DNA segments is initiated by the reaction of template DNA with oligonucleotide primers flanking the region to be amplified and is accomplished by a thermostable DNA polymerase. This methodology has been widely used in the diagnosis of infectious agents (14). However, PCR requires the use of radiolabeled probe for the detection of positive amplification reactions in the presence of low viral copy numbers (1, 9).

Multiple strategies have been described to circumvent the use of radioisotopes to detect PCR-amplified products (19, 21, 29). Gel electrophoresis with ethidium bromide staining does not reach sufficient sensitivity levels for detection of HIV-1, unless reamplification of PCR-amplified material is done with a nested set of primers (2). We have recently devised an enzyme immunoassay (EIA) for the quantitation of HIV-1 DNA amplified by the PCR (7).

We report here an improved and simplified version of this assay for HIV-1, designated PCR-EIA, in which the utilization of biotin-labeled RNA probes introduces an additional specificity step. PCR-amplified DNA is hybridized in solution with a complementary biotinylated RNA probe. Labeled DNA-RNA hybrids are then quantitated in a microtiter-plate EIA. The application of this methodology for HIV-1 detection in PBMC is compared here with a standard isotopic assay, the oligomer hybridization assay.

MATERIALS AND METHODS

Patients and sample preparation. A total of 108 specimens from 94 men seropositive for HIV-1 were randomly selected from stored PBMC kept at −70°C. PBMC were collected between August 1989 and November 1990. As part of an ongoing longitudinal study, 14 patients had two sequential samples drawn 5 to 7 months apart. Fifty-three patients (62 samples) had an asymptomatic HIV-1 infection, 22 (25 samples) had AIDS-related conditions, and 19 (21 samples) had AIDS. Lymphocytes from individuals without any risk factor for HIV-1 infection (n = 20) or with negative serology tests for antibodies against HIV-1 (n = 15) were included as negative controls. Serum samples had been initially screened by EIA for HIV-1 antibodies, and reactive specimens were confirmed by standard assays at the Laboratoire de Santé

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Publique de Québec (LSPQ) (16). The project had the approval of the ethics committee of the hospital, and informed-consent forms were signed by all of the patients.

Blood specimens were processed immediately after sampling. PBMC were isolated from fresh blood by Ficoll-Hypaque gradient centrifugation. Cells were washed three times in phosphate-buffered saline (10 mM phosphate buffer [pH 7.2], 150 mM NaCl) (PBS). The cell pellet was resuspended in a solution of 20 mM Tris (pH 8.3). Cell counts were determined in a counting chamber, and samples were kept at −70°C until they were processed for DNA amplification as described below.

Frozen cell suspensions were thawed and lysed by the addition of Tween 20 and Nonidet P-40, each at a final concentration of 0.4% (vol/vol). Samples were treated with proteinase K (final concentration, 250 μg/ml) for 2 h at 45°C and then heat denatured at 95°C for 10 min. Cell lysates were stored at −70°C until tested.

Oligonucleotide primers. The oligonucleotide primers were synthesized by the phosporamidite method. Sequences of primers were as follows: SK19, 5′-ATCCTGGGATTAAAT AAAATAGTAAGAATGTATAGCCCTAC (bp 1134 to 1174); SK38, 5′-ATAATCCACCTATCCCAGTAGGAGAAAT (bp 1090 to 1117); SK39, 5′-TTTGGTCCTTGTCTTATGTCCA GAATGC (bp 1177 to 1204); O1, 5′-GGGCAAATGGTAC ATCAGGCCATATCAC (bp 753 to 780); O2, 5′-TTTTAC CTCCTGTGAAGCTTGCTCGGCT (bp 1250 to 1277); N1, 5′-TTAATACGACTCACTATAGGGTAGAAGAGAAGGC TTTCAGC (bp 811 to 830); N2, 5′-TTGGTCCTTGTCTTAT GTCC (bp 1184 to 1203); N3, 5′-TTAATACGACTCACTAT AGGGTTATAAAAGATGGATAATCC (bp 1118 to 1137); N4, 5′-TGGTAGGGCTATACATTCTTA (bp 1156 to 1176).

The underlined sequences (primers N1 and N3) are those of the T7 RNA polymerase promoter (7, 35). The base pair numbers are from Wain-Hobson et al. (37). Primer pairs SK38/SK39 (28) and O1/O2 (8) amplified DNA segments of the *gag* gene of HIV-1 of 115 bp (bp 1090 to 1204) and 525 bp (bp 753 to 1277), respectively. The DNA fragment generated by amplification of HIV-1 with N1/N2 (bp 811 to 1203) was nested within the segment amplified with O1/O2, while sequences generated by amplification with N3/N4 (bp 1118 to 1176) were nested within the DNA segment generated with SK38/SK39. These nested DNA fragments were used as templates for the synthesis of biotinylated RNA probes (see below).

PCR. Cell preparations were thawed, and 100,000 to 250,000 lysed PBMC were subjected to PCR. Amplifications were performed in a 100-μl volume containing 2.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% (wt/vol) gelatin (Gen Amp PCR buffer II; Perkin-Elmer Cetus, Norwalk, Conn.), 0.5 μM each primer, 200 μM each deoxynucleotide triphosphate, and 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq from Perkin-Elmer Cetus and *Taq* from Amersham Corp., Oakville, Ontario, Canada). Samples were overlaid with 100 μl of mineral oil and amplified in a DNA thermal cycler heat block (Perkin-Elmer Cetus) through 30 cycles of denaturation at 94°C for 1 min, primer reannealing at 55°C for 1 min, and enzymatic DNA synthesis at 72°C for 1 min. To complete the synthesis of amplified products, a 10-min extension step at 72°C was introduced at the last cycle.

All procedures for sample preparation and PCR included the following measures to avoid cross-contamination between samples and contamination of reagents or samples with PCR-amplified products or positive controls (24). Initial processing of samples was performed with disposable pipettes in a biological hood that was not used for any other procedures related to PCR. Reagents and samples were mixed in sterile microcentrifuge tubes in another biosafety cabinet by using positive displacement pipettes (Mandel Scientific Co., Lachine, Quebec, Canada) or pipettors dedicated solely to PCR with filter pipette tips (USA Scientific Plastics Inc., Ocala, Fla.). All reagents were aliquoted before use. A negative buffer control was included in each PCR run. Products after PCR were handled in another laboratory.

Preparation of RNA probe. Specific RNA probes were transcribed from DNA segments generated by amplification with the nested sets of primers N1/N2 or N3/N4 (see above). A 100-pg amount of HIV-1 cloned in pSP64 (kindly provided by J. Campione from Health and Welfare Canada, Ottawa, Canada) was amplified with each nested set of primers. A single-stranded biotinylated RNA probe was prepared by incubation of 10 μl (±500 ng) of PCR products for 2 h at 40°C with 40 U of T7 RNA polymerase in the presence of 0.5 mM biotin-11-UTP (Enzo Inc., New York, N.Y.) under standard conditions (40 mM Tris [pH 7.5], 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 100 U of RNasin, 0.5 mM each rATP, rGTP, rCTP) (7, 8, 27). To enable digestion of the template and primers, the reaction mixtures were incubated for 20 min at 37°C with 2 U of DNase I. Reactions were stopped with 10 mM EDTA and by inactivation at 95°C for 5 min. Labeled RNA was separated from unincorporated bio-11-UTP by Sephadex G-25 chromatography with NAP-5 columns (Pharmacia, Uppsala, Sweden) by using diethylpyrocarbonate-treated water with 1% sodium dodecyl sulfate (SDS) as the elution buffer. SDS was included as an RNase inhibitor. The probe was aliquoted and stored at −20°C until use to prevent probe degradation by repeated freezing and thawing.

Nonisotopic solution hybridization assay with EIA for DNA-RNA hybrids for detection of PCR products. The nonisotopic solution hybridization assay for the detection of amplified DNA was performed as described previously (8, 11, 36). Briefly, 60 μl of the PCR mixture was mixed with 60 μl of hybridization buffer containing 4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7]), 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4), 2 mM EDTA, and biotinylated RNA probe from N1/N2 at 1 ng per ml or biotinylated RNA probe from N3/N4 at 100 ng/ml. These mixtures were heated to 100°C for 3 min and hybridized for 30 min at 78°C for the RNA probe generated with N1/N2 and heated to 60°C for the RNA probe generated with N3/N4. Samples were cooled to room temperature, and 10 μl of 10% Triton X-100 was added. Aliquots (50 μl) were tested in duplicate wells of clear microtiter plates (96 U-bottom wells; Falcon 3911 Microtest 111, Becton Dickinson, Oxnard, Calif.). These plates had been previously coated for at least 24 h and no more than 1 week at 4°C with 50 μl of an affinity-purified polyclonal goat antibody against biotin (Sigma Chemical Co., St. Louis, Mo.) per well at a concentration of 1 μg/ml in 0.06 M carbonate buffer (pH 9.6). Before use, the plates were washed six times with PBS–0.05% Tween 20 (PBST). After the addition of samples, the plates were incubated for 1 h at 37°C. After another washing step with PBST, 50 μl of a solution containing the Fab′ fragment of an anti-DNA-RNA monoclonal antibody conjugated to β-D-galactosidase, diluted to 0.050 μg/ml in PBST–0.5% gelatin, was added to each well. The properties of the monoclonal antibody have been described previously, and the monoclonal antibody was the generous gift of Robert Carrico, Miles Laboratories, Elkhardt, Ind. (4, 40). After

incubation for 1 h at 37°C, the plates were washed six times with PBST and 0.1 mM 4-methylumbelliferyl β-D-galactoside (Boehringer Mannheim Biochemicals, Laval, Quebec, Canada) in PBS with 1 mM $MgCl_2$ and 50 μg of bovine serum albumin per ml was added (50 μl per well). The amount of fluorescent methylumbelliferone generated by the enzymatic degradation of substrate after incubation at 37°C was measured in fluorescent units (FU) in a microtiter-plate fluorometer (Titertek Fluoroskan I; emission wavelength, 450 nm; detection wavelength, 365 nm). The background fluorescence activity of an empty well was subtracted from values recorded in the other wells (blank). A sample was considered positive if its mean fluorescent value exceeded that of the mean reactivity of a negative control plus 5 standard deviations (SD).

Detection of amplified HIV-1 DNA by radioactive oligomer hybridization (23). HIV-1 amplified with SK38/SK39 was detected with SK19. This oligonucleotide was 5'-end-labeled with T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (>5,000 mCi/mmol; Amersham) to a specific activity of $>10^9$ cpm/μg (33). A 10-μl volume of a mixture containing 0.2 pmol of end-labeled probe, 24 mM NaCl, and 4 mM EDTA was mixed with 30 μl of PCR products (20). After denaturation at 100°C for 5 min, this mixture was incubated at 55°C for 30 min. Hybridization mixtures were cooled on ice, and 5 μl of a loading buffer containing 0.01% bromophenol blue, 10% sucrose, and 1× TBE (90 mM Tris-borate buffer, 2 mM EDTA) was added. Portions (30 μl) of these mixtures were loaded in wells of a vertical 12% polyacrylamide gel. Electrophoresis was carried out for 8 h at 50 V in 1× TBE. The gel was enclosed in plastic wrap and exposed to a Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) with one intensifying screen for 12 h at −20°C.

RESULTS

Design of the nonisotopic assay for the detection of amplified DNA. In this study, we evaluated a novel methodology for the detection of HIV-1 proviral DNA amplified with PCR. The principle and optimization of PCR-EIA have been described previously (8). This assay has also been used for the detection of *Chlamydia trachomatis* (3) and human papillomavirus type 16 (6a) in clinical samples. Two sets of primers are selected for PCR-EIA. The outer set of primers is used exclusively on samples from patients to amplify viral sequences. Another set of primers, designated the nested set, is used to amplify a stock of HIV-1 DNA for the sole purpose of synthesizing a biotinylated RNA probe for the solution hybridization and EIA for biotinylated DNA-RNA hybrids.

The RNA probe is synthesized from DNA sequences of a segment contained within the fragment amplified with the outer set of primers without overlapping with the sequences of the latter primers. This location of the inner fragment is mandatory to prevent the probe from reacting with unincorporated oligonucleotide primers (8). For each set of nested primers, one primer included sequences of the T7 RNA polymerase promoter at its 5' end. The amplified products thus contained the double-stranded promoter and could be used as a template in a transcription reaction with T7 RNA polymerase and biotinylated UTP to generate a single-stranded biotinylated RNA probe (3, 7, 8, 34).

DNA amplified with the outer set of primers was hybridized in solution with the biotinylated RNA probe. The DNA-RNA hybrids formed were captured on microtiter plates coated with an antibiotin antibody. After unbound nucleic acids were removed by washing, specific hybrids bound to the solid phase were detected by their reactivities with anti-DNA-RNA monoclonal antibody conjugated to β-D-galactosidase and a fluorogenic substrate. The antibody specifically recognizes the structure of DNA-RNA hybrids but exhibits little reactivity with single- or double-stranded DNA or single-stranded RNA (4, 11, 36).

Amplification primers and probes. The primers and probe used for the detection of proviral DNA were derived from a conserved area in the *gag* gene of HIV-1. The DNA fragments generated with each primer pair were analyzed by gel electrophoresis and are depicted in Fig. 1. To evaluate the performance of PCR-EIA on clinical samples, three assays were compared. In the first assay, a PCR with SK38/SK39 was used and amplified products were detected with radiolabeled SK19 in the oligomer hybridization assay (PCR-OH). The second assay combined PCR with SK38/SK39 and detection of amplified material with an RNA probe synthesized with the nested primers N3/N4 (PCR-EIA,SK). The hybrid between single-stranded RNA probe and amplified DNA with SK38/SK39 was 59 bp in length. We have previously demonstrated that the length of DNA-RNA hybrids influences the endpoint sensitivity of the EIA for DNA-RNA hybrids (8). Since this might limit the sensitivity of the PCR-EIA,SK system, a third assay using the primer set O1/O2 and a 393-bp RNA probe generated with nested primers N1/N2 (PCR-EIA,O) (7, 8) was used in this study.

Sensitivity of PCR-EIA. The sensitivities of both PCR-EIAs were assessed by testing serial dilutions of a plasmid containing the genome of HIV-1. Each dilution was amplified in separate reactions with each outer set of primers in 1 μg of placental DNA per reaction mixture. Amplified material was tested with the respective biotinylated RNA probe. Hybridization conditions and parameters of the EIA for DNA-RNA hybrids have been carefully studied previously (7, 8, 11, 36). Plates were left standing for substrate degradation at 37°C for 3 h for PCR-EIA,O and for 18 h for PCR-EIA,SK. The low background noise of the shorter RNA probe allowed for such a prolonged incubation time and was necessary to obtain good signals in the EIA for DNA-RNA hybrids. The background noise of each PCR-EIA assay was the reactivity of the probe with amplified human placental DNA. As shown in Table 1, each PCR-EIA assay could detect 10 copies of HIV-1 DNA above the detection cutoff (mean reactivity of background noise of the probe plus 5 SD) (Table 1), although the signal was stronger at this endpoint with the longer DNA-RNA hybrids in spite of the shorter substrate incubation time.

Detection of HIV-1 DNA in PBMC samples. To investigate the performance of PCR-EIA in the detection of proviral DNA in PBMC samples, 108 specimens from seropositive patients and 20 samples from individuals without risk factor for HIV-1 were studied in all three PCR assays. The reactivities of the negative samples in PCR-EIA,SK ranged from 4.5 to 22.5 FU, with a mean reactivity of 11 ± 5.2 FU for a detection cutoff of 37 FU [(5 × 5.2 FU) + 11 FU = 37 FU] (Fig. 2). The reactivities of negative samples tested in PCR-EIA,O ranged from 2 to 24.5 FU, with a mean of 14.7 ± 8.6 FU, for a detection cutoff of 57.7 FU [(5 × 8.7 FU) + 14.7 FU = 57.7 FU]. There was 100% concordance between the assays that used the radiolabeled and nonisotopic probes for SK38/SK39 products. The results of PCR-OH from representative samples are depicted in Fig. 3.

Results of tests with the PCR-EIAs of all of the samples from seropositive patients and the seronegative controls are shown in Fig. 2. Samples from seropositive patients exhib-

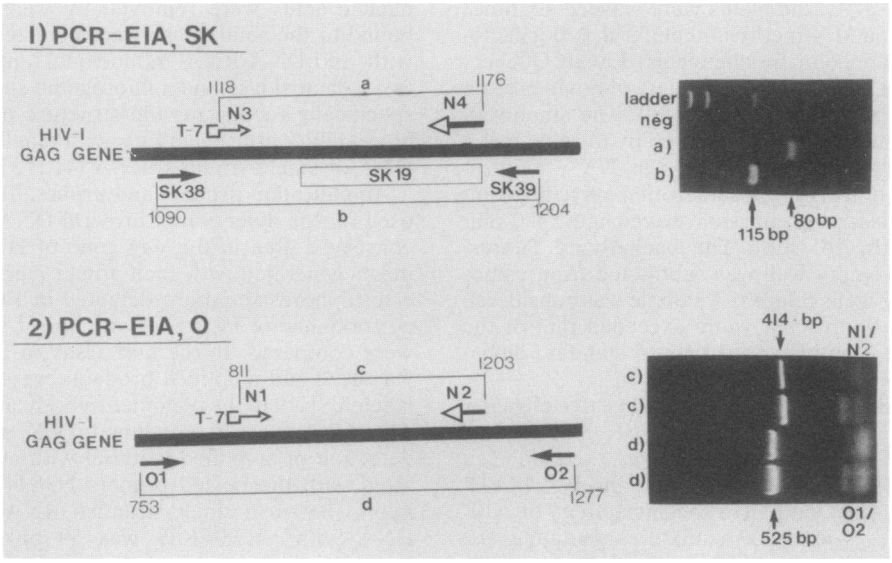

FIG. 1. Location of primers on HIV-1 genome. Positions of the different primers for the HIV-1 *gag* gene are indicated by arrows. SK38, N1, O1, and N3 are complementary to the viral plus strand, whereas SK39, O2, N2, and N4 are complementary to the viral minus strand. The gel electrophoresis of the DNA fragments generated with each primer pair are shown on the right side of the figure. Symbols and abbreviations: T-7, T7 RNA polymerase promoter; ⊏→, nested primer with the T7 promoter at its 5′ end; ◁━, nested primer on the minus strand; ━▶, outer sets of primers; SK19, internal probe for PCR-OH; ladder, molecular size ladder of *Hae*III-digested ΦX174 replicative form DNA; neg, a buffer control. Lanes: a, products of amplification with N3/N4; b, products of amplification with SK38/SK39; c, duplicates of products of amplification with N1/N2; d, duplicates of products of amplification with O1/O2. Positions of amplified fragments are in base pairs as described by Wain-Hobson et al. (37).

ited a broad range of reactivities, from 24.1 to 645 FU in PCR-EIA,SK and from 40 to 1,006 FU in PCR-EIA,O. The mean reactivity of positive samples tested in PCR-EIA,SK of 175.2 ± 116.3 FU was significantly lower than that obtained with PCR-EIA,O (424.7 ± 283.2 FU) ($P < 0.01$; Mann-Whitney U test [39]).

Of the 108 specimens from seropositive patients, 104 were positive with PCR-EIA,SK (sensitivity, 96.3%) and 104 were positive with PCR-EIA,O (sensitivity, 96.3%). Only one sample was negative with a combination of primers. Three samples were positive with SK38/SK39 and negative with O1/O2, while three samples were negative with SK38/SK39 and positive with O1/O2. The discordant samples came from two patients with AIDS-related conditions and four asymptomatic patients. These samples were not necessarily weak positives (Fig. 2).

TABLE 1. Detection of HIV-1 proviral DNA with PCR-EIA[a]

Initial amt of HIV-1 DNA (no. of molecules)	HIV-1 detected (FU) (mean ± SD)	
	SK38/SK39	O1/O2
10^4	418.5 ± 34.6	715 ± 37
10^3	133.0 ± 29.7	515 ± 11
10^2	55.0 ± 1	163 ± 2
10^1	25.0 ± 2	40 ± 2
10^0	11.8 ± 3.1	18 ± 2
Probe	13.8 ± 2.2	15 ± 3

[a] Samples containing a constant genomic DNA content of 1 μg and decreasing amounts of HIV-1 DNA copies were subjected to amplification with two primer pairs, SK38/SK39 and O1/O2. The amount of amplified material was quantitated in the solution hybridization and EIA for DNA-RNA hybrids with each respective biotinylated RNA probe (see Materials and Methods). Results are expressed as the mean FU ± SD of duplicate testings. The fluorescent activity of substrate alone was subtracted from each value. The background reactivity of the RNA probe was determined by reactivity of the probe with amplified material from placental DNA. The detection cutoff for each test was the background reactivity of the probe plus 5 SD (for SK38/SK39, 13.8 + 11 = 24.8 FU; for O1/O2, 15 + 15 = 30 FU).

All of the discordant samples and positive samples were analyzed with a primer pair (KM29/RS42) that amplifies a region of the β-globin gene (31). This procedure allowed for the evaluation of the integrity of cellular DNA and for the presence of inhibitors of *Taq* polymerase. An amount of cells equivalent to that used for amplification of HIV-1 was amplified with KM29/RS42. The amplified product was subjected to agarose gel electrophoresis and stained with ethidium bromide (Fig. 4). The samples that were positive with both primer pairs for HIV-1 showed a specific band of 536 bp when tested for β-globin. The PCR for β-globin was weakly positive in one discordant sample and negative for the others. The weakly positive sample for β-globin turned negative when the sample was diluted. However, when the other discordant samples were diluted to 1/10 of the initial amount of PBMCs and retested, they became positive for the B-globin gene. The presence of an inhibitor in these samples was suspected.

All of the samples from seropositive patients that were negative or discordant upon initial testing for HIV-1 were diluted 10-fold and retested in PCR-EIA,SK and PCR-EIA,O. Two of the four samples that were negative in PCR-EIA,SK became positive, and two of the four samples that were negative in PCR,O became positive. The sample negative with both primer pairs remained negative (data not shown). When results with diluted samples are included, the revised sensitivity of PCR-EIA,SK was 98% (102 of 104 samples) and that of PCR-EIA,O was also 98% (102 of 104 samples). Each assay still failed to detect one sample that was positive in the other assay.

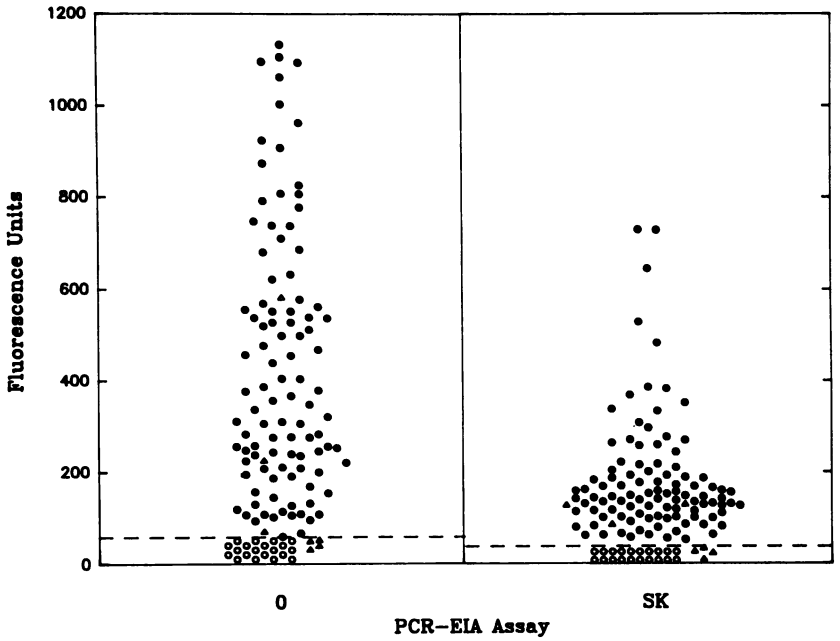

FIG. 2. Detection of HIV-1 DNA in clinical samples by using PCR-EIA. PBMC lysates were amplified with primers SK38/SK39 (SK) and primers O1/O2 (O). Amplified material was detected with a biotinylated RNA probe as described in Materials and Methods. Symbols: ○, seronegative controls; ●, seropositive patients; △, seropositive patients negative in one PCR-EIA test (six patients) or in both PCR-EIA tests (one patient). The detection cutoff (– – –) was the mean reactivity of samples from seronegative patients plus 5 SD (for PCR-EIA,SK = 37 FU; for PCR-EIA,O = 57.7 FU).

DISCUSSION

A practical assay system combining the extreme sensitivity of PCR and the convenience of biotin-labeled probes and the EIA format was developed for HIV-1 detection. Optimization of parameters of the solution hybridization and EIA for DNA-RNA hybrids has been reported elsewhere (8). These studies have revealed that the hybridization reaction in solution is completed in 30 min. The rapidity of the reaction can be attributed to the use of single-stranded RNA probe and favorable reassociation kinetics of homogeneous hybridization reactions. The hybridization reaction can be performed under very stringent conditions, with a reannealing temperature of 78°C. The EIA for DNA-RNA hybrids is linear over 2 logs of target DNA and can detect 5 pg of complementary DNA (8). The signal in the EIA depends on the length of DNA-RNA hybrids, but hybrids of 25 bp can be detected, although with less sensitivity (8).

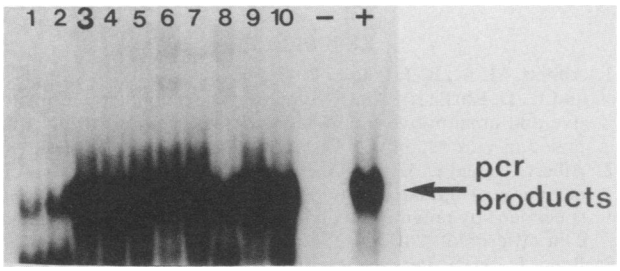

FIG. 3. Detection of HIV-1 DNA in PBMC by using PCR-OH. Cell lysates were amplified with primers SK38/SK39 and reacted with ^{32}P-labeled SK19. Hybridized material was run on a 12% polyacrylamide gel, and autoradiography was done at −20°C for 12 h. Lanes: 1 to 10, samples from antibody-positive individuals; −, buffer control; +, 1 pg of a plasmid containing HIV-1.

Our first assay using the monoclonal antibody against DNA-RNA hybrids for HIV-1 DNA detection differed from the one presented here (7). In that assay, we utilized the monoclonal antibody to capture and quantitate DNA-RNA hybrids formed after reaction with an unlabeled RNA probe. Since any DNA-RNA or RNA-RNA hybrids already present before PCR could react in the EIA, preamplified material had to be tested concurrently. The occurrence of background noise in some preamplified samples (2a, 6b) invalidated the assay for those samples. To avoid testing preamplified samples and to increase the specificity of the EIA, biotin-labeled probes were used.

The incorporation of additional sequences appended to the 5′ end of a primer, such as the T7 RNA polymerase promoter, has been described previously and does not interfere with amplification of target sequences (3, 7, 34, 35). For probes shorter than 500 bases, the signal is proportional to probe length, and, thus, assays that use long probe sequences give a higher signal (8). The requirement to prolong the substrate incubation step in the EIA when short probes are used, as in the case of PCR-EIA,SK, makes the assay less convenient.

PCR-EIA avoids the manipulation of radioactive material while achieving sensitivity levels comparable to those of assays with ^{32}P-labeled probes. EIAs are widely used and thus provide a convenient assay system for measuring PCR-amplified material. PCR-EIA,O can be completed in 5 h and yields quantitative results. The substrate incubation period can be shortened by using a fluorogenic substrate for alkaline phosphatase (10). For those laboratories without access to a fluorometer, colorigenic substrates can replace fluorogenic substrates, but a longer incubation period for substrate

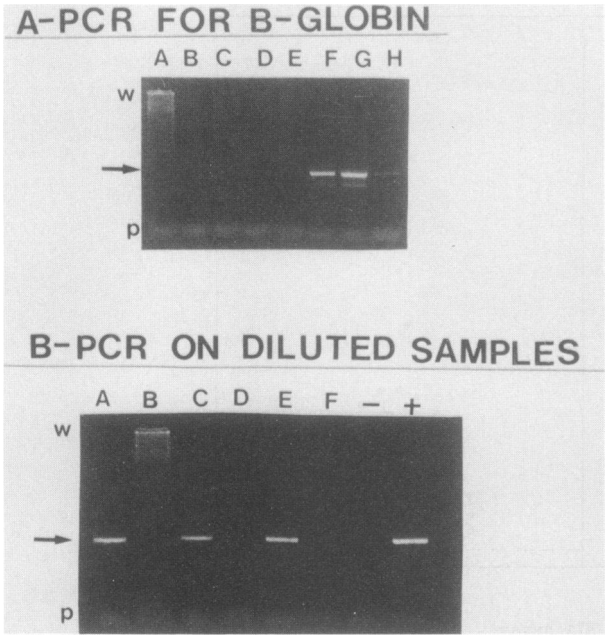

FIG. 4. Representative results of amplification analysis for β-globin in PBMC samples. Amplifications were done with primers for β-globin (see text), and 10-μl aliquots were run on 2% agarose gels stained with ethidium bromide. A specific band of 536 bp represents a positive reaction for β-globin (arrows on the left of gels). Panel A illustrates the reactivities of some samples. Lanes: A to E, samples with discordant results between primer pairs for HIV-1 *gag* gene; F and G, samples amplified with both sets of primers for HIV-1; H, a sample discordant between primer pairs for HIV-1 but weakly positive for the β-globin gene. w, position of the wells; p, location of the unincorporated primers. Panel B illustrates the removal of inhibitory effect of samples on PCR with dilution of samples previously unreactive for β-globin. Lanes: A, C, and E, diluted samples (dilution ratio, 1/10) from lanes B, D, and F, respectively. –, a negative buffer control; +, a positive control.

degradation is required to reach comparable levels of sensitivity (10). The method is versatile and can be applied to any PCR assay by selecting a nested set of primers to prepare an RNA probe.

The EIA is quantitative (7), but, because accurate measurements of cell counts by cell sorting were not available and because the number of amplified PBMC varied from patient to patient, we have not attempted to use PCR-EIA to quantitate viral load. For this purpose, the number of cycles must be in the range in which the rate of amplification is exponential, which is usually less than 25 cycles unless target DNA is in minute amounts. A quantitative PCR assay must also control for factors in the sample that influence the efficiency of PCR and for the variability of the amplification process (9, 12). Internal standards amplified with the same primers used for the target sequences represent the ideal system for such use of PCR (38).

When applied to detection of HIV-1 DNA in PBMC, PCR-EIA compared favorably with PCR-OH. As described in other studies in which the *gag* gene of HIV-1 was amplified, proviral DNA was detected in nearly all seropositive patients by PCR (7, 17, 18, 26, 29). The sensitivity of PCR was improved when a combination of two primer pairs was used on clinical specimens.

One problem encountered by multiple investigators has been the inconsistent detection of HIV-1 sequences with different pairs of primers (6, 9, 28). A low viral load could explain some discrepant results. In samples with low viral copy numbers, interference by nonspecific amplification or suboptimal amplification conditions could lead to false-negative reactions for one primer pair. The variable efficiency of primers to bind to target DNA from diverse strains could also explain some false-negative results. Mismatches located at the 3′ ends of primers are the most likely to inhibit the amplification process (25). Inosine-substituted primers have been used to prevent failures of PCR due to 3′-end mismatches (22). A loss of integrity of DNA during sample manipulation could also be the cause of one false-negative result (Fig. 4A, lane H) (30).

Coamplification of a cellular gene can control for the integrity and amount of DNA input in the reaction (30). In this study, most samples that were discordant between primer pairs with PCR-EIA were negative in a PCR for the B-globin gene. The effect of inhibition of PCR seemed to be reversible with sample dilution, which suggests that degradation of DNA was not a factor here. Inhibitors of *Taq* polymerase copurifying with DNA could play a role in cases of seropositive patients negative by PCR. A high salt concentration (5) and hemoglobin from lysed erythrocytes (15) are known to inhibit *Taq* polymerase, but most inhibitor substances in clinical samples have not been characterized. In some cases, inhibitors could be removed from samples by a combination of boiling and gel filtration of samples (13). Amplification of a cellular gene has been proposed as a way to identify those samples that contain inhibitors of PCR. However, it has not been firmly established that the degree of inhibition of amplification is uniform for different sets of primers. Also, the degree of inhibition of PCR for low-copy-number targets could be difficult to evaluate by using control reactions detecting abundant sequences that reach saturation of the amplification process early in PCR. We are presently conducting further studies on the samples which were found to contain inhibitory substances.

PCR-EIA is an efficient and practical method to detect trace amounts of nucleic acid in biological samples. It has the potential to make PCR more amenable for use in diagnostic laboratories and for large-scale epidemiological studies.

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