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Inhibition of Human Immunodeficiency Virus Gene Amplification by Heparin

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Gene amplification of virus-specific sequences is widely used as a method to detect or confirm human immunodeficiency virus (HIV) infection. In this study we used an enzyme-linked affinity assay to quantify polymerase chain reaction products from whole blood, plasma, and separated mononuclear cells collected in the presence of four common anticoagulants: acid citrate dextrose, sodium EDTA, potassium oxalate, and sodium heparin. Attenuation of the product signal was observed after amplification of nucleic acid extraction from whole blood, washed mononuclear cells, and plasma from specimens collected in sodium heparin. These inhibitory effects on gene amplification could be reversed with heparinase. The addition of as little as 0.05 U of heparin completely inhibited amplification of an HLA-DQa sequence from placental DNA. We conclude that heparin can cause attenuation or inhibition of gene amplification. Acid citrate dextrose and EDTA, which lack inhibitory activity, are the most appropriate anticoagulants for clinical blood samples when polymerase chain reaction amplification is anticipated.

The polymerase chain reaction (PCR) is used with increasing frequency to diagnose infectious or genetic diseases. Gene amplification depends on a thermostable DNA polymerase from *Thermus acquaticus* (*Taq*) (21); the enzymatic characteristics and properties of *Taq* DNA polymerase have recently been reviewed. Certain concentrations of KCl, Mg^{2+}, ammonium chloride, urea, dimethyl sulfoxide, dimethylformamide, formamide, and sodium dodecyl sulfate have inhibitory effects on *Taq* DNA polymerase activity (10).

Clinical research by PCR is often performed with DNA and RNA obtained from whole blood, peripheral blood mononuclear cells (PBMCs), or plasma collected in the presence of an anticoagulant. Blood samples collected in the presence of heparin have been shown to yield decreased quantities of cellular DNA (12), and an inhibitory effect of heparin on gene amplification has recently been described (2). We investigated the suitability of four anticoagulants (heparin, EDTA, acid citrate dextrose [ACD], and potassium oxalate) for the purposes of nucleic acid amplification. Here we define and quantitate the inhibitory effect of heparin on PCR by using an enzyme-linked affinity assay for PCR product detection and quantitation.

MATERIALS AND METHODS

Sample preparation. Informed consent was obtained from all subjects prior to sample collection. Whole blood was drawn from five human immunodeficiency virus (HIV) antibody-positive patients and three HIV-seronegative controls and was collected in evacuated blood collection tubes (Becton Dickinson) in the presence of the three anticoagulants ACD solution A (0.15 ml/ml), sodium heparin (14.3 U/ml), and EDTA (1 mg/ml) and in tubes with no additives. To model the presence of HIV RNA and DNA in peripheral blood, reconstruction experiments were performed in which 10^6 HIV-infected U1 cells were added to 10 ml of HIV-seronegative whole blood collected in tubes containing no additives or anticoagulants, including potassium oxalate (2 mg/ml). PBMCs were isolated by using lymphocyte separation medium (Organon Teknika) and washed twice with 10 ml of phosphate-buffered saline. Serum and plasma were removed after 10 min of centrifugation at 800 × *g*.

Nucleic acid extraction and reverse transcription. Cellular DNA from whole blood collected in the presence of ACD, EDTA, oxalate, and heparin was obtained by the method described by Higuchi (14). Cellular RNA from washed PBMCs and cell-free RNA from 200 μl of HIV-infected patient plasma or serum were extracted by a guanadinium thiocyanate-phenol-chloroform method (6). Isolation of cellular DNA from washed PBMCs and whole blood was performed by a standard phenol-chloroform extraction and ethanol precipitation method (18). For PCR amplification of cDNA, 1 μg of RNA was reverse transcribed with Moloney-murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) by a previously published protocol (16).

Gene amplification. Oligomers used for HIV PCR included SK38, SK39, and SK19. Oligomers used for HLA-DQ were GH26 and GH27, all of whose sequences have been published previously (13, 20). Biotinylation of primer SK38 and horseradish peroxidase labeling of probe SK19 were done as described previously (17).

Amplification of a HIV *gag* sequence from U1 cell DNA, patient cDNA, and patient DNA was carried out in a 100-μl reaction volume containing 50 mM KCl, 20 mM Tris hydrochloride (pH 8.4), 2.5 mM $MgCl_2$, 0.02% gelatin, 1 mM (each) deoxynucleoside triphosphates (Pharmacia), 50 pmol of each primer, and 2.5 U of Amplitaq DNA polymerase (Perkin Elmer-Cetus). The mixture was overlaid with 50 μl of lightweight mineral oil, and 30 cycles of amplification were performed in a DNA thermal cycler (Perkin Elmer-Cetus) with the following program: 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a 10-min extension at 72°C.

Amplification of a HLA-DQ sequence from 1 μg of purified placental DNA (Sigma) was performed in the presence

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of a 10-fold dilution series of preservative-free heparin (50 to 0.0005 U per reaction tube; Lyphomed). In some experiments in which heparin was added to placental DNA, a 10-fold excess of enzyme (25 U of *Taq* DNA polymerase) was added to PCRs or heparin and placental DNA were incubated with 7.4 U of heparinase II (Sigma) per reaction tube (in 1× PCR buffer [50 mM KCl, 2.5 mM $MgCl_2$, 20 mM Tris hydrochloride, pH 8.4, 0.02% gelatin]) for 30 min at 37°C prior to PCR. PCR parameters were the same as those described above for HIV *gag* primer pairs. PCR product from reactions with human HLA-DQ primers were evaluated by agarose gel electrophoresis and ethidium bromide staining of 10 μl of PCR product.

Enzyme-linked affinity assay. To detect and quantitate PCR product, avidinated 2.54-μm-diameter polystyrene beads (Eastman Kodak) were mixed 1:1 with a blocking solution containing 5× Denhardt solution, 0.5% gelatin, and 50 μg of sheared herring sperm DNA per ml and incubated overnight at 4°C. The PCR product (5 μl) and 5× SSPE (saline, sodium phosphate, EDTA) (45 μl) were heated to 95°C for 5 min and then cooled on ice to denature the sample. One picomole of SK19-HRP (an oligonucleotide probe complementary to the amplified sequence) in 5× Denhardt solution was added to the denatured PCR product and hybridized for 1 h at 42°C. Blocked avidinated beads (100 μl; approximately 150 pmol of avidin) was added to each well of a 1.2-μm-thick loprodyne membrane bottom plate (Pall Biosupport). The beads were washed with phosphate-buffered saline by suspension and filtering on a vacuum filtration holder (Millipore). Hybridized PCR product was added to a well containing beads, and a capture reaction was allowed to take place for 20 min. The bead-target-oligomer probe complex was then washed with phosphate-buffered saline. The chromogenic substrate *o*-phenylenediamine (Sigma) was added to each well for 10 min. The reaction was stopped with 2 N H_2SO_4 and vacuum filtered into a clear polystyrene microtiter plate (Costar). The $A_{490/405}$ was read on a plate reader (Dynatek). A negative absorbance cutoff of 0.135 was previously defined by taking the mean absorbance of 15 samples from HIV-seronegative patient (0.087 ± 0.016) plus three standard deviations.

RESULTS

The ability to amplify the HIV *gag* sequence from cellular DNA and reverse-transcribed RNA obtained from cells collected in different anticoagulants was assessed in reconstruction experiments in which 10^6 HIV-infected U1 cells (provided by AIDS Research and Reference Reagent Program, National Institutes of Health) were added to HIV-seronegative whole blood, collected in the presence of sodium heparin, ACD, EDTA, and potassium oxalate. The U1 cell line contains two integrated copies of HIV DNA per cell (8). In these experiments, the enzyme-linked affinity assay demonstrated an attenuation of the signal from DNA processed from whole blood or DNA and RNA from separated mononuclear cells collected in heparin, whereas it did not demonstrate attenuation of the signal from DNA from cells collected in EDTA, ACD, and oxalate (Fig. 1).

Blood samples obtained from five HIV-seropositive patients collected in ACD, EDTA, or sodium heparin were processed to obtain (i) RNA extracted from plasma, (ii) RNA extracted from PBMCs, and (iii) DNA from PBMCs. The RNA from cells or plasma was reverse transcribed, and all specimens were amplified by using the *gag* gene primers. In each patient sample, the enzyme-linked affinity assay results

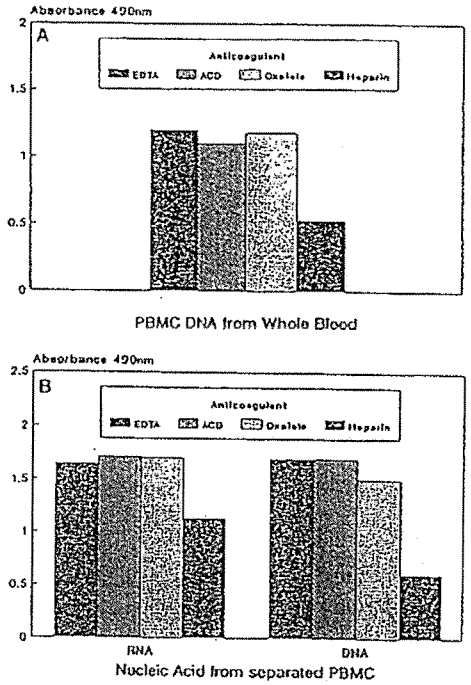

FIG. 1. Absorbance values for HIV *gag* gene PCR products from U1 cellular DNA and RNA. HIV-infected U1 cells were added to HIV-seronegative whole blood in the presence of different anticoagulants. (A) Results for DNA extracted from whole blood. (B) Results for DNA and RNA from separated mononuclear cells.

demonstrated suppression of the signal in the samples collected in heparin, whereas it did not demonstrate suppression in serum or plasma samples collected in EDTA or ACD (Fig. 2A). The PCR product signal for cellular RNA was decreased from blood collected in heparin as compared with that from blood collected in EDTA and ACD (Fig. 2B), and there was a marked decrease in the signal from cellular DNA collected in the presence of heparin that was reversed when extracted DNA was preincubated with heparinase prior to PCR (Fig. 2C).

Heparin was shown to inhibit DNA amplification directly, as shown in Fig. 3. In these experiments, human placental DNA was amplified by using HLA-DQ primers, and the PCR product was visualized by ethidium bromide staining of agarose gels. The addition of ≥0.05 U of heparin per reaction suppressed the presence of a visible band in ethidium bromide-stained agarose gels in a dose-dependent fashion. When a 10-fold increase of *Taq* polymerase (25 U per reaction) was added to each reaction in a series containing heparin, there was some reversal of inhibition, and when samples were incubated with heparinase prior to PCR, there was a modest reversal of the inhibitory effect.

DISCUSSION

Heparin appears to be a potent inhibitor of gene amplification reactions when it is added directly to PCRs or when

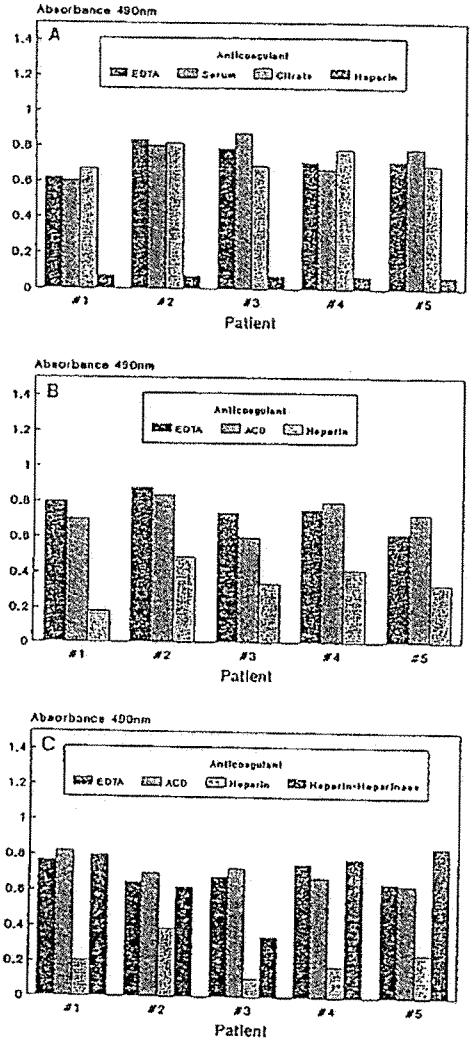

FIG. 2. Absorbance values for HIV *gag* gene PCR products from samples from five HIV-positive patients. (A) Results for plasma and serum collected in the presence of EDTA, ACD, heparin, and no additives. (B) Results for cellular RNA from separated HIV-infected mononuclear cells collected in the presence of EDTA, ACD, and heparin. (C) Results for DNA from separated mononuclear cells to which heparinase was added to heparin-containing samples.

nucleic acid is extracted from cells or plasma collected in heparinized tubes. The inhibitory effects of heparin did not prevent qualitative detection of HIV sequences from cellular nucleic acid, but there was a marked quantitative decrease in PCR products, as measured by an enzyme-linked affinity assay. The most dramatic inhibitory effect was observed when RNA was extracted from plasma and reverse transcribed and PCR was performed. In these studies, plasma collected in heparin demonstrated a very low signal compared with that of plasma collected in EDTA or ACD.

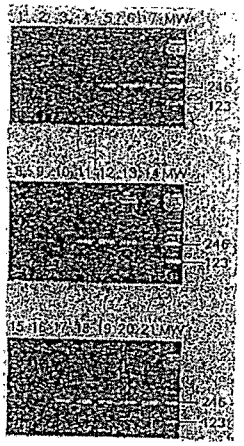

FIG. 3. Inhibition of gene amplification by heparin. One microgram of purified placental DNA was amplified with HLA-DQ primers GH26 and GH27 (242 bp) in the presence of preservative-free heparin. The ethidium bromide-stained agarose gel contained a 1/10th volume of amplification reactions. Tenfold dilutions of heparin were added prior to PCR. In addition to heparin, some samples contained a 10-fold excess (25 U) of *Taq* polymerase, and other samples were preincubated with 7.4 U of heparinase prior to PCR. Lanes 1 to 6, Heparin alone (50 to 0.0005 U per reaction); lane 7, placental DNA, no heparin; lanes 8 to 13, heparin (50 to 0.0005 U per reaction) and 25 U of *Taq* polymerase per reaction; lane 14, placental DNA and 25 U of *Taq* polymerase, no heparin; lanes 15 to 20, heparin (50 to 0.0005 U per reaction) and 7.4 U of heparinase per reaction; lane 21, placental DNA and 7.4 U of heparinase, no heparin; MW, molecular size markers (numbers to the right are in base pairs).

Previous studies have shown that heparin competitively inhibits several different cellular DNA polymerases (9), simian sarcoma virus reverse transcriptase (7), and HIV reverse transcriptase (15). In addition, heparin has been shown to bind to the HIV envelope glycoprotein gp120 (4). Heparin is also used in the purification of DNA and RNA polymerases by affinity chromatography on heparin-Sepharose (3, 11, 19). DNA polymerases bound to heparin-Sepharose can be eluted with salt, suggesting a reversible inhibition (7). Heparin inhibition of certain DNA polymerases can be overcome by increased KCl concentrations (3), increased enzyme, or additional template primer (7). In contrast, reverse transcriptase appears to be less sensitive, in that simian sarcoma virus reverse transcriptase is inhibited to a lesser degree (7), and HIV and Moloney-murine leukemia virus reverse transcriptases are inhibited only by very high concentrations of heparin (1).

Use of heparin in the collection of blood specimens may pose additional problems. Differences in the stability of cellular nucleic acid collected in the presence of different anticoagulants observed by Gustafson et al. (12) indicate that the stability and yield of cellular DNA collected in ACD are far superior to those of samples collected in heparin. In addition, heparin has been shown to inhibit some restriction endonucleases (5).

The effect of heparin noted in this study can potentially introduce a variable decrease in the amount of PCR product obtained. Collection of blood in heparin is unlikely to affect the overall sensitivity of PCR as a method for detecting the presence of HIV sequences. However, heparin may lead to a reduction in PCR product when clinical specimens are obtained for quantification of virus or proviral DNA by PCR. In conclusion, our findings demonstrate that heparin can inhibit gene amplification and may not be removed after nucleic acid extraction and suggest that blood samples for PCR should be collected in ACD or EDTA.

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