

## Detection of Human Immunodeficiency Virus Type 1 (HIV-1) in Urine Cell Pellets from HIV-1-Seropositive Individuals

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**Fresh urine pellets from human immunodeficiency virus type 1 (HIV-1)-seropositive individuals were examined for the presence of the HIV-1 genomic sequence and gene products. By using the polymerase chain reaction technique, HIV-1 DNA proviral sequences were detected in 53 of 80 (66.25%) fresh urine pellets from HIV-1-seropositive individuals, while urine pellets from all 24 healthy heterosexual controls were negative. HIV-1 RNA in urine pellets was detected by reverse transcriptase polymerase chain reaction in 2 of 43 (4.7%) HIV-1-seropositive individuals. In addition, HIV-1 p24 core antigen was demonstrated in 3 of 80 urine pellets from HIV-1-seropositive individuals by enzyme-linked immunosorbent assay. Moreover, HIV-1 p24 core antigen and HIV-1 RNA were shown in the cellular component of urine pellets from HIV-1-seropositive individuals by immunohistochemical staining and in situ hybridization. These results indicate that HIV-1 can be present in urine pellets from HIV-1-infected individuals.**

Human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, has been isolated from several body fluids of infected individuals (6, 9, 10, 23, 25). We have previously shown the prevalence of specific immunoglobulin G antibody to HIV-1 in the urine of HIV-1-seropositive individuals by enzyme-linked immunosorbent assay (ELISA) and Western immunoblot assay (3). In addition, we recently reported the presence of HIV-1 proviral DNA sequences in urine cell pellets from HIV-1-infected individuals (14). We describe here the detection of HIV-1-specific proviral DNA and RNA in centrifuged fresh urine pellets from some HIV-1-seropositive individuals. Moreover, the mononuclear cells present within these urine pellets contained HIV-1 DNA, RNA, and p24 core protein, as demonstrated by in situ hybridization, immunohistochemical staining, and ELISA.

### MATERIALS AND METHODS

Blood and urine samples were collected from 80 randomly selected HIV-1-seropositive homosexual males attending a clinic at New York University Medical Center. Among them, 19 were healthy, asymptomatic HIV-1-seropositive individuals, 17 had AIDS-related complex, 40 had AIDS-related Kaposi's sarcoma, and 4 had AIDS associated with an opportunistic infection. Twenty-four healthy HIV-1-seronegative heterosexual male employees of the New York University Medical Center served as normal controls. Urine samples from all patients were found to be within normal limits by routine urinalysis. In addition, the blood urea nitrogen and creatinine levels in all patients were in the normal range.

Mononuclear cells from peripheral blood were separated by gradient centrifugation with Ficoll-Hypaque. Cell pellets from ~100-ml fresh urine specimens were obtained by centrifugation at  $1,200 \times g$  at 4°C for 5 min and were then washed twice with phosphate-buffered saline (PBS). The DNA and RNA samples were extracted from urine pellets of different specimens from each individual as described previ-

ously (16). DNAs were dialyzed in dialysis bags with 10 mM Tris-Cl-1 mM EDTA (TE) overnight and were then precipitated with ethanol.

The polymerase chain reaction (PCR) amplification method was then performed on these specimens with DNA and 10 pmol of each of the HIV-1 *gag* gene primers (SK38 and SK39) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100 µg of gelatin per ml, 2.5 U of *Taq* polymerase, and 225 µM (each) deoxynucleoside triphosphates. Samples were subjected to 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). The PCR products were analyzed by either slot blot hybridization or liquid hybridization (1). For liquid hybridization, one-fourth of the amplified product was boiled for 5 min and then hybridized with the <sup>32</sup>P-end-labeled HIV-1 *gag* gene probe SK19 ( $2.5 \times 10^5$  cpm per reaction for 2 h at 55°C in a solution containing 0.15 M NaCl). The reactions were then analyzed in a 12% polyacrylamide gel. Autoradiograms were obtained after exposure to Kodak X-ray film at -70°C.

Urine specimens were randomly collected from 49 of the 80 HIV-1-seropositive individuals. RNAs were extracted, and reverse transcriptase (RT)-PCR was performed as we described previously (2). Briefly, total RNA was incubated with 4 U of RNase-free DNase (Promega Biotec, Madison, Wis.) at 37°C for 45 min. The enzyme was subsequently heat inactivated (at 95°C for 5 min), and reverse transcription was carried out in a volume of 20 µl which contained 50 pmol of the downstream primer SK39, 225 µM (each) deoxynucleoside triphosphates, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl<sub>2</sub>, 2 µg of nuclease-free bovine serum albumin, and 200 U of moloney murine leukemia virus RT (Bethesda Research Laboratories, Gaithersburg, Md.) that was incubated at 42°C for 30 min. The cDNA was then amplified as in the procedure described above.

**Immunohistochemical staining.** Freshly centrifuged urine pellets were washed twice with PBS, and the pellets were then smeared on glass slides. After air drying, the slides were fixed in acetone at room temperature for 20 min. Endogenous peroxidase activity was eliminated by a 15-min wash in 3% hydrogen peroxide. The slide smears were then

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sequentially incubated in normal goat serum at room temperature for 30 min, mouse anti-p24 monoclonal antibody (NEN/Dupont, Billerica, Mass.) was then added, and the slide smears with the antibodies added were then incubated overnight at room temperature. Biotinylated goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was then added for 2 h at room temperature, and then horseradish peroxidase-streptavidin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added and the slides were incubated for an additional 30 min at room temperature. Between each incubation the slide smears were washed twice for 5 min each time in PBS. As a substrate solution we used 0.2 mg of 3,3-diaminoberydine (Polysciences Inc., Warrington, Pa.) per ml in PBS (pH 7.4) supplemented with 0.1% hydrogen peroxide. Finally, the slides were counterstained with hematoxylin and examined by microscopy (8, 19). HIV-1-infected H9 cells served as positive controls, while noninfected H9 cells, normal control urine cell pellets smeared onto slides, and HIV-1-infected cells incubated with normal mouse serum served as negative controls.

**In situ hybridization.** Fresh urine pellets were fixed on poly-L-lysine-coated slides and were then treated with 0.2 N HCl for 20 min at room temperature. The slides were washed and then treated with proteinase K (1 µg/ml) for 5 min at 37°C. After rinsing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the slides were treated with 0.25% (vol/vol) acetic anhydride in triethanolamine for 10 min and were then washed in 2× SSC at room temperature for 5 min. The slides were covered with prehybridization buffer (2× SSC containing Denhardt's solution [0.02% Ficoll-400, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone-40] and 10% dextran sulfate in 20 mM sodium phosphate [pH 7.2]) for 1 h at 37°C. After washing, the slides were covered by the hybridization solution, which contained 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 1 mg of yeast tRNA per ml, 100 µg of salmon sperm DNA per ml, and 5 × 10<sup>5</sup> cpm of <sup>35</sup>S-labeled HIV-1 RNA (NEN/Dupont) incubated at 37°C overnight. The slides were rinsed in 2× SSC twice at room temperature and were then washed again in 2× SSC at 55°C for 1 h. The slides were then washed with 50% formamide-0.6 M NaCl-100 mM Tris-1 mM EDTA at room temperature. After washing, the autoradiographic detection of hybrids was carried out by dipping the slides in Kodak NTB<sub>2</sub> emulsion diluted 1:1 with distilled water containing 0.3 M ammonium acetate. The urine pellet-smear slides were allowed to air dry and were then placed in a desiccation chamber at 4°C for 1 to 4 weeks. The slides were subsequently developed in Kodak D19 developer and were stained with hematoxylin and eosin (5, 13, 15).

**ELISA for HIV-1 p24 antigen in urine pellets.** Eighty urine pellets were collected from fresh urine by centrifugation at 1,200 × g. The pellets were then suspended in 200 µl of PBS and were lysed by repeated freezing and thawing. After microcentrifugation at 10,000 × g, the supernatants were then assayed for the presence of HIV-1 p24 core protein antigen by an antigen capture enzyme immunoassay (Abbott Laboratories, North Chicago, Ill.).

## RESULTS

HIV-1 DNA proviral sequences were detected by PCR in 53 of 80 fresh urine pellets from HIV-1-seropositive individuals. The urine pellets were found to contain HIV-1 proviral DNA sequences in 13 of 17 (76%) patients with AIDS-related complex, 26 of 40 (65%) patients with AIDS and Kaposi's

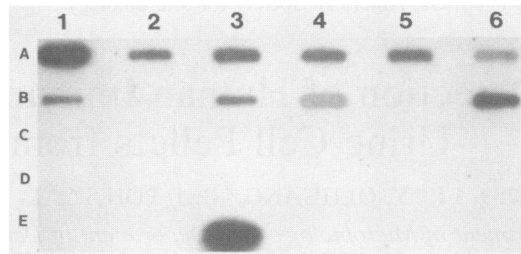


FIG. 1. Representative PCR analysis of peripheral blood mononuclear cell and urine cell pellets. DNAs were amplified with primer pair SK38-SK39 and were detected with the SK19 probe as described in the text. The slot blots contained PCR products from the following samples: A1 to A6, peripheral blood mononuclear cells from HIV-1-seropositive patients; B1 to B6, fresh urine pellets from HIV-1-seropositive patients; C1 to C6 and D1 to D6, peripheral blood mononuclear cell and urine cell pellets, respectively, from healthy controls; E1, human placenta DNA; E2, primer pair control; E3, HIV-1-infected H9 cells as a positive control.

sarcoma, 3 of 4 (75%) patients with AIDS and an opportunistic infection, and 11 of 19 (58%) healthy, asymptomatic individuals. Urine pellets from all 24 healthy controls were negative for HIV-1 DNA by PCR (Fig. 1).

HIV-1 RNA in the urine pellets was detected by the RT-PCR method (2). Among the fresh urine pellets from 43 HIV-1-seropositive individuals, 2 (4.7%) showed positive signals by RT-PCR (Fig. 2).

To confirm that the urine pellets contained HIV-1, immunohistochemical staining and in situ hybridization were performed on the same urine pellets. As shown in Fig. 3 and 4, p24 antigen was detected in the cellular component of the fresh urine pellets by monoclonal antibody immunohistochemical staining. The p24 antigen was not detected in any of the fresh urine pellets from HIV-1-uninfected healthy individuals. The antibody staining for p24 was found to be confined to only a few of the mononuclear cells found in the urine pellets. Only 3 of 80 urine specimens from the HIV-1-seropositive individuals were found to be p24 antigen positive by ELISA performed on the supernatants of lysed urine pellets. The level of the p24 core protein detected in these specimens ranged from 31 to 1,000 pg/ml.

By the in situ hybridization method, use of the <sup>35</sup>S-labeled HIV-1 probe resulted in a strong hybridization signal in HIV-1-infected H9 lymphocyte cultures, and positive signals were also detected in the cells that were present in 2 of 10 urine pellets from HIV-1-seropositive individuals (Fig. 4).

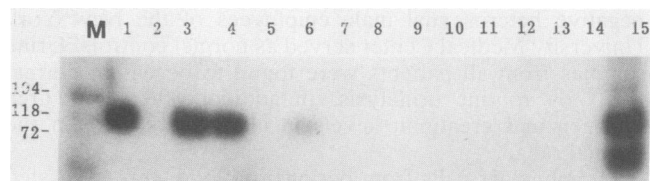


FIG. 2. Representative results of RT-PCR of urine cell pellets. Lanes 1 to 3 and 4 to 6, urine pellets from two HIV-1-seropositive individuals; lanes 7 to 9 and 10 to 12, urine pellets from two HIV-1-seronegative individuals; lanes 1, 4, 7, and 10, DNA PCR; lanes 2, 5, 8, and 11, PCR after digestion with DNase I; lanes 3, 6, 9, and 12, RT-PCR; lanes 13 and 14, negative control using human placenta DNA and primer pairs, respectively; lane 15, RT-PCR from HIV-1-infected H9 cells as positive controls; lane M, molecular mass markers (in base pairs).

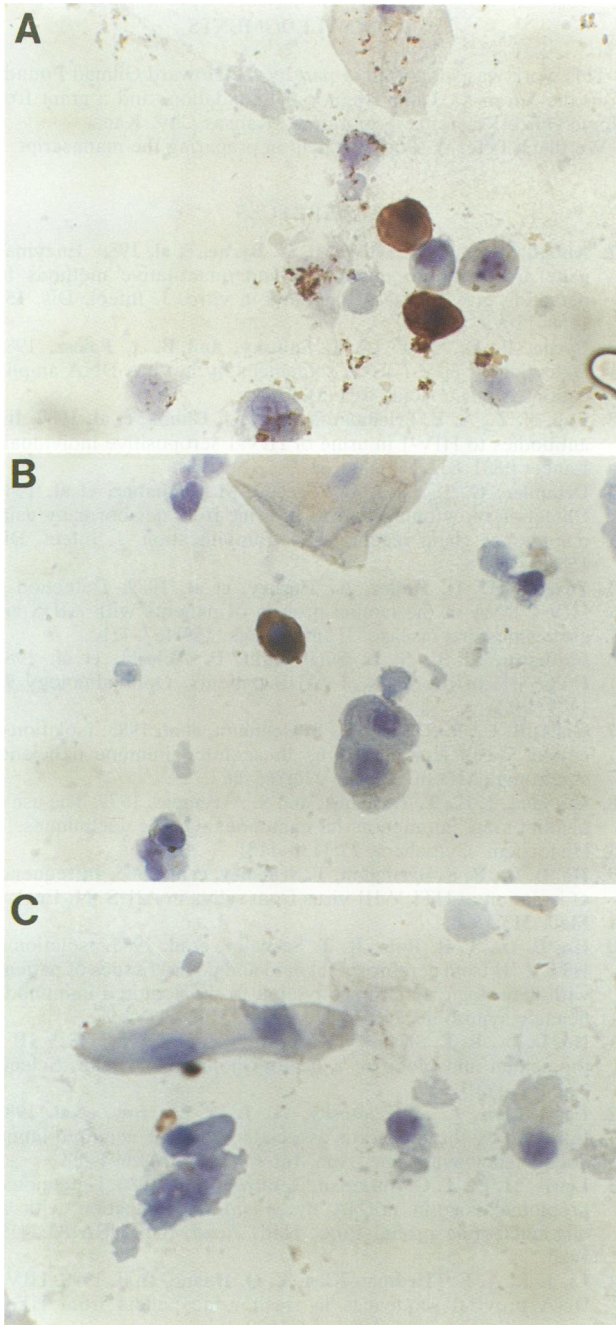


FIG. 3. Immunostaining of urine cell pellets with monoclonal antibody to HIV-1 p24. (A and B) Urine cell pellets from HIV-1-seropositive individuals; positive cells showed brown staining. (C) Urine cell pellets from an HIV-1-seronegative individual. (Magnification,  $\times 260$ ).

#### DISCUSSION

It has been demonstrated previously that normal human urine frequently contains small amounts of mononuclear cells and erythrocytes. Detection of infectious organisms such as JC virus, cytomegalovirus, human papillomavirus, and *Leptospira* spp. in urine has been reported previously (4, 18, 20, 24). HIV-1 has previously been isolated from various body fluids from HIV-1-seropositive individuals, including blood, semen, tears, saliva, cerebrospinal fluid, breast milk,

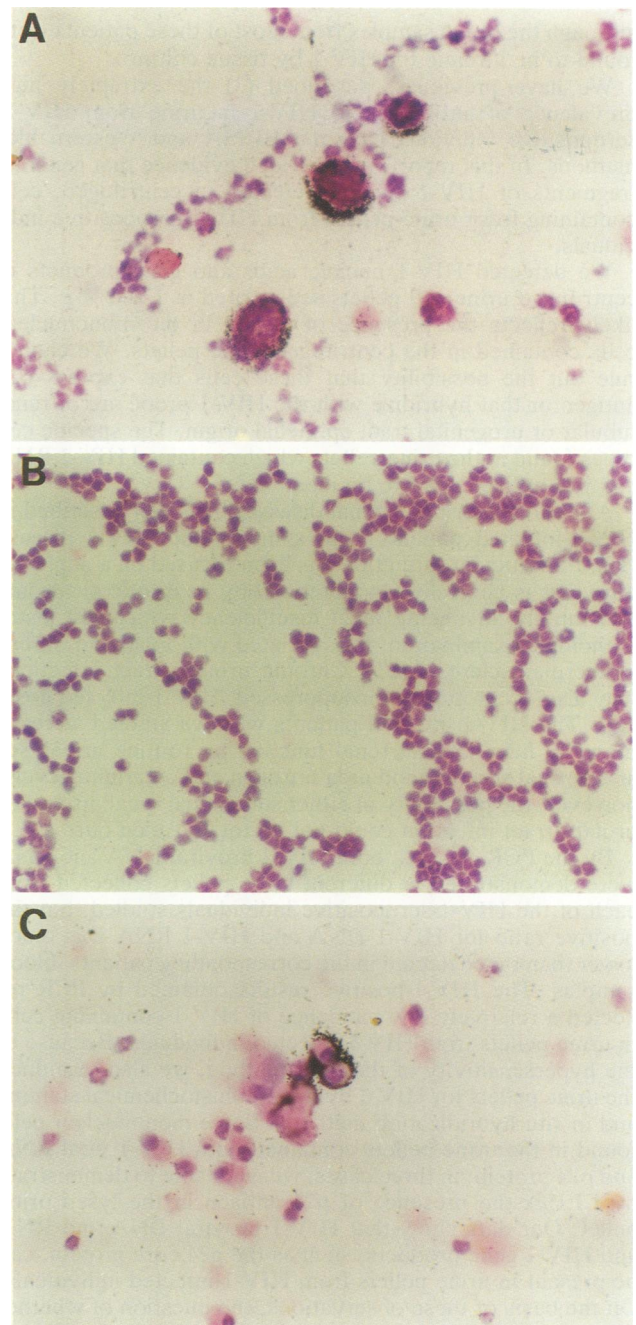


FIG. 4. In situ hybridization of urine cell pellets from HIV-1-seropositive individuals with  $^{35}\text{S}$ -labeled HIV-1 antisense probe. (A) Positive control from HIV-1-infected H9 cells. (B) Negative control from HIV-1-uninfected H9 cells. (C) Fresh urine pellets from patients with AIDS showing a strong hybridization signal in a few mononuclear cells. Magnification,  $\times 260$ .

and cervical secretions (6, 7, 9-11, 23, 25). With the increasing incidence of HIV-1 infection, the question of whether infectious HIV-1 can be detected in urine from infected individuals becomes increasingly important, since urine could serve as a vehicle for HIV-1 transmission. Levy et al. (12) detected HIV-1 in one of five urine specimens examined. However, Skolnick et al. (22) were unable to detect HIV-1 in urine from 50 HIV-1-seropositive individuals,

although the blood samples from most of these patients were found to be viremic for HIV-1 by tissue culture.

We have previously described (3) the extremely high prevalence of antibodies to HIV-1 in urine from HIV-1-seropositive individuals by the ELISA and Western blot methods. In this report, we provided evidence that genomic fragments of HIV-1 can be detected in centrifuged, cell-containing fresh urine pellets from HIV-1-seropositive individuals.

We detected HIV-1 nucleic acids and gene products in centrifuged urine cell pellets sedimented at  $1,200 \times g$ . This likely reflects the presence of HIV-1 in the mononuclear cells contained in the centrifuged urine pellets. We cannot rule out the possibility that these cells that express p24 antigen or that hybridize with the HIV-1 probe are of renal tubular or urogenital tract epithelial origin. The specific cell types found in the urine pellets which contained HIV-1 RNA and p24 antigen need to be identified.

A broad spectrum of renal diseases has been described in HIV-1-infected patients with symptomatic HIV-1 disease (17). In particular, attention has been focused on a nephropathy that is clinically characterized by nephrotic syndrome and rapidly advancing renal insufficiency which, by histopathologic examination, is associated with focal and diffuse glomerular sclerosis (21). Chronic urinary tract infections may also occur in immunosuppressed, HIV-1-infected hosts (17). The HIV-1-infected patients who we studied were all found to have normal renal function by routine urinalyses and normal serum blood urea nitrogen and creatinine levels; however, the possibility of either subclinical renal disease or urinary tract infection could not be totally ruled out.

By the PCR method, both HIV-1 proviral DNA and RNA were demonstrated in different urine pellets collected from each of the HIV-1-seropositive individuals studied, but the positive ratio for HIV-1 DNA and HIV-1 RNA was much lower than that detected in the corresponding patients' blood samples. The HIV-1-positive results obtained by PCR reflected a relatively low incidence of HIV-1-containing cells in urine pellets from HIV-1-infected individuals. Because of the hypersensitivity of the PCR method, we also examined the urine pellets for HIV-1 by immunohistochemical staining and *in situ* hybridization methods. Some mononuclear cells found in the urine pellets contained both HIV-1 viral RNA and p24 protein; in three cases, we were able to demonstrate by ELISA the presence of p24 antigen in the lysed urine pellet. Our data show that HIV-1 proviral DNA and RNA and HIV-1 gene products, such as the p24 core protein, can be present in urine pellets from HIV-1-infected individuals. On the basis of these observations, the question of whether urine is potentially an infectious body fluid in the HIV-1-infected host is raised. In their attempts to isolate infectious HIV-1 from urine, other investigators have not been able to culture infectious HIV-1 from the urine of HIV-1-seropositive individuals (22). However, our results demonstrate that HIV-1 genomic sequences can, in fact, be detected in centrifuged urine cells pellets from some seropositive individuals.

By the PCR technique, it is theoretically possible to detect HIV-1 proviral DNA sequences in the centrifuged pellets of fresh urine specimens from HIV-1-seropositive individuals. Previous studies in which PCR was used to detect HIV-1 sequences have focused almost exclusively on blood samples from HIV-1-infected individuals. The demonstration that HIV-1 proviral DNA and RNA sequences can be detected in urine cell pellets by the PCR technique might be useful as a noninvasive test for confirming HIV-1 infection.

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