Detection and localization of HIV-1 DNA in renal tissues by *in situ* polymerase chain reaction

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Summary. The localization of HIV-1 DNA in renal tissues is critically important for understanding pathogenesis of HIV-associated nephropathy (HIVAN), but the clarification has been technically challenging. We applied *in situ* polymerase chain reaction (IS-PCR) to human renal tissues to demonstrate viral entry into the renal epithelial cells *in vivo*.

To test the specificity of this method and to determine the cell types infected, we used IS-PCR followed by *in situ* hybridization (ISH) and IS-PCR followed by immunohistochemistry and histochemical counterstains.

Brief 2 hour fixation in 4% paraformaldehyde had 92.9% sensitivity and 100% specificity for detection of viral DNA in renal biopsies of HIVAN patients, compared to 70.8% sensitivity and 66.7% specificity in renal biopsies fixed overnight in 10% formalin. Under optimized conditions, the only signals detectable in HIV-1 seronegative cases were false positives attributable to renal tubular apoptosis. In HIVAN cases, positive signal was observed in podocytes, parietal cells, renal tubular cells, and interstitial leukocytes. Immunohistochemical co-labeling for pan-T cell and macrophage markers revealed that the interstitial leukocytes with positivity for HIV-1 DNA included both T cells and macrophages. Application of ISH after IS-PCR showed the same distribution of signal as observed using IS-PCR alone, confirming the specificity of the technique.

IS-PCR is a powerful technique to detect viral DNA in human tissue sections, but requires proper use of negative controls to set optimal fixation, protein digestion, and amplification conditions.

Key words: HIV-1 DNA, HIV-associated nephropathy, *In situ* PCR, Kidney

Introduction

While conventional PCR has been helpful to identify virus and to determine viral load within the kidney, the *in situ* polymerase chain reaction (IS-PCR) technique has the advantage of localizing gene products to specific renal compartments or cell types with high sensitivity. Kimmel et al. (1993) have identified HIV gene products in microdissected renal compartments. Unlike microdissection followed by conventional PCR, IS-PCR obviates possible contamination by unwanted cell types and allows the colocalization of various proteins and RNAs. However, because of problems inherent in the use of tissue sections, it is much more difficult to set optimal conditions for IS-PCR than conventional PCR.

HIV-associated nephropathy (HIVAN) is a renal disorder caused by renal epithelial infection by HIV-1. HIVAN patients present proteinuria and progressive renal failure and manifest pathologic features of collapsing glomerulopathy and tubular microcysts. Several groups have demonstrated the presence of HIV sequences in the tissues of HIV-1 seropositive patients (Bagastra and Pomerantz, 1993; Embertson et al., 1993; Nuovo et al., 1993; Patterson et al., 1993; Zavollus et al., 1994; Bagastra et al., 1996; An et al., 1999). The possibility of HIV-1 infection of human kidney was suggested by *in vitro* experiments showing that renal cells are permissive to viral infection (Green et al., 1992; Ray et al., 1998). Cohen et al. (1989) first demonstrated HIV-1 nucleic acid in renal tubular cells of patients with HIVAN using DNA *in situ* hybridization (ISH) technique. However, these ISH results were difficult to duplicate, however, due to low expression levels and requirements for brief fixation time (Bruggeman et al., 1997). In addition, there are few monoclonal antibodies available that bind HIV-1 in tissue sections with a high degree of sensitivity and specificity. Because monoclonal antibodies recognize a single epitope, they may not be sensitive enough to detect low viral copy number in intrinsic renal cells. In previous work, we successfully localized HIV-1 DNA in renal tissues of

patients with HIVAN using IS-PCR (Bruggeman et al., 2000). In the current study, we examine a larger cohort of HIVAN patients and controls to address the issue of the sensitivity and specificity of this method. We applied IS-PCR followed by ISH to test the specificity of the experiments. Based on our data, optimized IS-PCR provides a useful tool to investigate the role of renal parenchymal infection by HIV-1 in the development of HIVAN.

Materials and methods

Clinical Materials

Renal tissues obtained by biopsy or at autopsy from HIV-1 seropositive patients with HIVAN (N=43) were studied. The mean ages were 41.1±1.4 years for the group with HIVAN. Their clinical profiles are summarized in Table 1. The study cohort included 29 African Americans, 8 Hispanics, and 1 Asian. At the time of biopsy, many patients were receiving highly active antiretroviral therapy (HAART).

To optimize the fixation conditions, three fixation protocols were examined. First, biopsy specimens were fixed routinely (at least overnight) with 10% buffered formalin (Group 1; 24 HIVAN cases). Second, autopsy specimens were fixed for 24-72 hours with either 10% buffered formalin or 4% paraformaldehyde, pH 7.4 (PFA) (Group 2; 5 HIVAN cases). Third, biopsy specimens were fixed for 2 hrs with 4% PFA (Group 3; 14 HIVAN cases). All fixed tissues were otherwise processed the same way. Following fixation, tissues were dehydrated in graded alcohols and embedded in paraffin. Sections were cut at 3 µm, and mounted on slides coated with 3-aminopropyltriethoxy silane (Sigma, St Louis, MO) followed by incubation at 60°C overnight to maximize tissue adhesion.

As case controls, we used renal biopsies from 28 HIV-1 seronegative cases (24 in Group 1 and 4 in Group 3) with a variety of glomerular, tubulo-interstitial or vascular diseases (Table 1).

DNA extraction and conventional PCR

Unstained paraffin-embedded tissues were scraped from slides into microcentrifuge tubes and DNA was extracted by the protocol of QIAamp Tissue Kit (QIAGEN, Valencia, CA), in which 0.5 mM Tris-HCl, pH 9.0 with 1.25 µg/ml tRNA was used in place of the elution buffer. Briefly, the microcentrifuge tubes were incubated with 0.2 mg/ml proteinase K solution at 37°C for 4 hrs. The DNA in the tissue lysate was then bound to the QIAamp spin column and eluted with 0.5 mM Tris-HCl, pH 9.0 with 1.25 µg/ml tRNA. The samples were RNase A-treated (0.1 µg/ml for 30 min at 37°C) followed by lyophylization to reduce volume to approximately 30 µl. DNA extracted from the samples (10 µl of the above column eluate) was added to an amplification mixture including buffer C (Invitrogen,

Carlsbad, CA), 250 ng of each primers, 200 µM dNTP (Roche Molecular Biochemicals, Indianapolis, IN), and 1 µl AmpliTaq (Perkin Elmer, Foster, CA) in a total volume of 50 µl. The sense primer for HIV-1 envelope DNA corresponded to base pairs (bp) 6827-6855 of pNL4-3 (5' TGTCCAAAGGTATCCTTTGAGCCAAT TCC 3') and the antisense primer to bp 7367 - 7341 of pNL4-3 (5' AGTAGAAAAATTCCCCTCCACAATTAA 3'). Position of the primers is specified relative to the predicted transcription initiation site. Forty-five cycles of PCR were performed on each sample with a thermal programmer (Perkin Elmer) as follows; denaturation at 94°C for 60 sec, annealing at 68°C for 30 sec, and extension at 72°C for 30 sec. As for negative controls, 25 ng of genomic DNA was applied in place of sample DNAs. For glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which is a pan-renal marker, DNA extracted from the samples (1 µl of the above column eluate) was added to the same amplification mixture. The sense primer for G3PDH corresponded to base pairs (bp) 586-605 (5' ACCACAGTCCATGCCATCAC 3') and the antisense primer to bp 1018 - 1037 (5' TCCACCA CCCTGTTGCTGTA 3'). Fifty cycles of PCR were performed on each sample with a thermal programmer (Perkin Elmer) as follows; denaturation at 94°C for 60 sec, annealing at 60°C for 45 sec, and extension at 72°C for 60 sec. Aliquots of the PCR products were then electrophoresed on a 2% agarose gel stained with ethidium bromide.

To confirm the PCR results for HIV-1 envelope DNA, Southern blotting was performed using a ^{32}P-labeled probe corresponding to pNL4-3 sequences internal to the PCR primers. Briefly, hybridization was performed at 42°C in Hybrisol (Intergen, Purchase, NY) followed by stringent washes at 60°C in 0.1 x SCC with 0.1% SDS.

DNA IS-PCR

Sections were deparaffinized over 2 days using

Table 1. Patient demographics.

	HIVAN	HIV seronegative
Number of patients	43	28
Age (mean, range)	41.1 (19-60)	45.7 (21 - 70)
Race		
African American	29	12
Caucasian	0	9
Hispanic	8	2
Asian	1	1
Unknown	5	4
Gender		
Male	32	18
Female	11	10
CD4 count < 200	15/26 (57.7%)	0
On anti-retroviral therapy?	10/20 (50.0%)	0

several exchanges of xylene and were rehydrated through graded alcohols. A 2-day deparaffinization time was chosen because preliminary data indicated that insufficient deparaffinization hindered staining. The sections were then treated with 0.02 M HCl for 10 min and permeabilized with proteinase K (1-5 µg/ml) for 1 hour at 37°C. After inactivation of this enzyme, the slides were washed twice in phosphate-buffered saline, pH 7.4 (PBS), for 10 min and air-dried. Prior to PCR amplification, the sections were treated with 10 µM dNTP with or without 0.1 U/µl Klenow enzyme (New England BioLabs, Beverly, MA) at 37°C for 30 min. The sections were pre-heated at 70°C prior to the PCR cycles to achieve a hot start. The PCR amplification was carried out using OmniSlide (Hybaid, Middlesex, UK). The reaction solution used for PCR contained the following: 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 mM digoxigenin-11-dUTP (Roche Molecular Biochemicals), 1 ng/ml of each primer, 0.05 U/µl DNA Taq polymerase (Gibco, Geitherburg, MD), and 50% self-seal reagent (MJ Research, Watertown, MA) in total volume of 100 µl. The solution was placed directly on the tissue section and covered with Frame-Seal Chambers (MJ research). The following cycles were applied: initial denaturation at 95°C for 2 min followed by the total 30 cycles of 95°C for 1 min, 68°C for 30 sec, and 72°C for 70 sec. The final extension was at 72°C for 7 min.

After completion of the PCR cycles, the sections were washed with the graded SSCs, incubated with 1% Blocking reagent (Roche Molecular Biochemicals) for 30 min at room temperature, and incubated with the alkaline phosphatase conjugated monoclonal antibody against digoxigenin Fab fragment (Roche Molecular Biochemicals) at 1:1000 dilution. After overnight incubation, the sections were washed three times for 5 min each in PBS. The alkaline phosphatase was visualized in red using SIGMA FAST (Sigma). HIV-1-infected cells display nuclear positivity. After stopping the reaction with distilled water, sections were lightly counterstained with methyl green or PAS to highlight renal basement membranes, including tubular, glomerular and vascular basement membranes.

As for negative controls for IS-PCR, either the DNA Taq polymerase step was omitted or samples were treated with DNase prior to the procedure. Amplification of the human ß-globin gene, a single copy gene, was performed as a positive control for IS-PCR, as follows: initial denaturation at 92°C for 2 min followed by 30 cycles of 92°C for 1 min and 65°C for 2 min. The final extension was at 65°C for 7 min. The sense primer for ß-globin corresponded to bp 14 - 33 (5' ACACAACTGT GTTCACTAGC 3') and the antisense primer to bp 123 - 104 (5' CAACTTCATCCACGTTCACC 3'). Kidney tissue from the transgenic murine model (Dickie et al., 1991), in which a deletion construct of the HIV-1 provirus pNL4-3 was used as a transgene, was also applied as a positive control.

The combined intensity and distribution of the *in situ* PCR results were determined on a scale of 0 to 3+ (0, absent; 1+, low intensity and <25% of cells stained; 2+, moderate intensity and <50% of cells; 3+, high intensity and >50% of cells stained) for the podocytes, parietal cells, tubular epithelial cells, intraluminal apoptotic cells, and interstitial cells. For each case, the histologic scoring was performed based on microscopic examination of the entire tissue section.

DNA ISH after IS-PCR

The specificity of the IS-PCR was tested by sequential ISH using a probe internal to the IS-PCR products with no overlap with the primers. The internal probe was made using a PCR technique as follows: The extracted DNA from pNL4-3 was added as a template to an amplification mixture including 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 mM digoxigenin-11-dUTP, 1 ng/µl of each primer, and 0.05 U/µl DNA Taq polymerase. The sense primer corresponded to bp 6911 - 6932 (5' TTCAATGGAACAGGACCATGTA 3') and the antisense primer to bp 7336 - 7318 (5' GTGCGTTAC AATTTCTGGG 3'). The following cycles were applied: initial denaturation at 94°C for 5 min followed by the total 30 cycles of 94°C for 1 min, 56°C for 30 sec, and 72°C for 45 sec. The final extension was at 72°C for 7 min. After the electrophoresis on a 2% agarose gel stained with ethidium bromide, the discrete band with 425-bp size was excised. The digoxigenin-labeled probe was purified using Wizard PCR Preps DNA Amplification System (Promega, Madison, WI) according to manufacturer's protocol. The yield was roughly estimated by comparing with the digoxigenin-labeled control in DIG DNA Labeling Kit (Roche Molecular Biochemicals).

The same procedures were applied for IS-PCR before hybridization except for the substitution of 4 µM biotin-14-dATP (Roche Molecular Biochemicals) for digoxigenin-11-dUTP in order to decrease the diffusion of amplified products (Matsuoka et al., 1998).

After amplification, hybridization was performed as follows: Frame-Seal Chambers were removed while dipping with 2 x SSC, and slides were fixed in 4% PFA for 20 min at room temperature. After post-PCR fixation, sections were washed twice for 5 min in PBS at room temperature, dehydrated in a graded ethanol series, and air-dried. The digoxigenin-labeled probe nested within the PCR amplicon was applied to each section at a concentration of 50 ng/100 µl in hybridization buffer (4 x SSC, 10% deionized formamide and 10% dextran sulfate) and the sections were then covered with Frame-Seal Chambers. The slides were denatured at 95°C for 5 min, and were hybridized for 12-16 hrs at room temperature on OmniSlide. Then, Frame-Seal Chambers were removed and the slides were washed in 2 x SSC at 45°C for 15 min, and 1 x SSC at 45°C for 15 min. The same procedure described above to detect digoxigenin-labeled probe was then applied.

As a negative control for ISH, the denaturation step was omitted. Moreover, irrelevant primers, M13 primers

(Invitrogen), were applied during IS-PCR for non-specific amplification prior to ISH.

Double labeling after IS-PCR by immunohistochemistry for CD3 or CD68

After amplification, immunohistochemistry was carried out. The sections were microwaved for 25 min and treated with 1% H_2O_2 in ethanol for 30 min to quench all endogenous peroxidase activity prior to immunostaining. Following blocking with 10% normal horse serum (Vector Labs, Burlingame, CA), the sections were stained with anti-pan T cells monoclonal antibody (CD3; DAKO, Carpinteria, CA) (1:50) and anti-macrophage monoclonal antibody (CD68; Vector Labs) (1:50) and incubated overnight at 4°C in a humidity chamber. Following PBS wash, sections were stained with biotinylated secondary horse anti-mouse antibody (1:100) (Vector Labs) for 30 min, followed by 3,3' diaminobenzidine solution containing 0.003 % H_2O_2. Sections were lightly counterstained with PAS and then mounted.

Negative controls for the immunohistochemical staining consisted of serial sections treated in the same way but for the substitution of PBS for primary antibody.

Statistical analysis

Statistical significance (p<0.01) was determined by Fisher's exact probability test using StatView Version 4.01 (Abacus Concepts Inc., Berkeley, CA).

Results

Conventional PCR

Figures 1A and 1C show the amplification products

for HIV-1 envelope DNA and G3PDH DNA respectively using DNA extracted from unstained paraffin sections in Group 3 (lanes 1-4) and genomic DNA (lanes 5-8). Lane 9 contains a negative control in which DNase was used to eliminate any DNA as template. In lanes 1-4, the PCR products can be clearly identified as intense bands corresponding to HIV-1 envelope DNA (541 bp
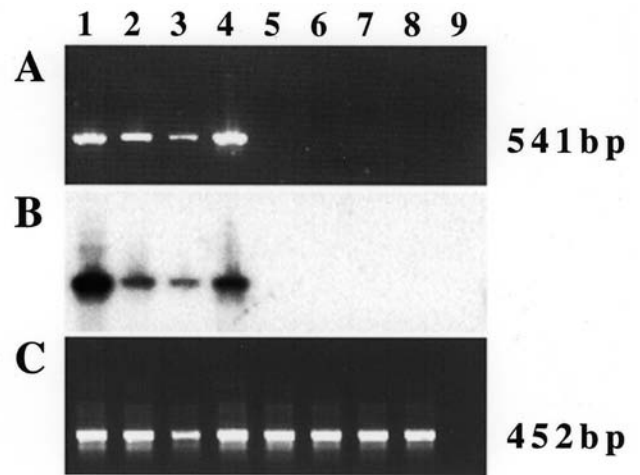
Fig. 1. The results of DNA amplification (PCR 40 cycles) by conventional PCR using DNA extracted from unstained paraffin sections of Group 3 patients with HIVAN (lanes 1-4) and genomic DNA controls (lanes 5-8) are shown. **Panel A** shows amplification products for HIV-1 envelope DNA and **panel C** shows amplification products for G3PDH. Lanes 1-4 contain PCR products for both HIV-1 envelope DNA (541 bp product) and G3PDH (452 bp product), whereas lanes 5-8 contain only the 452 bp product. Lane 9 is a negative control in which DNase was used to eliminate DNA as template. **Panel B** confirms the results using Southern blotting for HIV-1 envelope DNA. The molecular marker used was 100 bp ladder.

Table 2. Results of IS-PCR.

	PODOCYTES	PARIETAL EPITHELIUM	TUBULAR EPITHELIUM	"EPITHELIAL CELLS" *	INTRALUMINAL APOPTOSIS	INTERSTITIAL CELLS
Group 1 HIVAN n = 24	8/24 (33.3%) x = 1.0	3/24 (12.5%) x = 1.0	14/24 (58.3%) x = 1.21	17/24 (70.8%)	19/24 (79.2%) x = 1.0	9/24 (37.5%) x = 1.62
Group 1 HIV seronegative n = 24	0/24	0/24	4/24 (16.7%) x = 1.0	4/24 (16.7%)	12/24 (50%) x = 1.0	7/24 (29.2%) x = 1.0
Group 2 HIVAN n = 5	3/5 (60%) x = 1.33	4/5 (80%) x = 1.75	5/5 (100%) x = 1.8	5/5 (100%)	5/5 (100%) x = 1.2	5/5 (100%) x = 2.0
Group 3 HIVAN n = 14	7/14 (50%) x = 1.43	6/14 (42.9%) x = 1.33	12/14 (85.7%) x = 1.75	12/14 (85.7%)	8/14 (57.1%) x = 1.0	11/14 (78.6%) x = 1.18
Group 3 HIV seronegative n = 4	0/4 —	0/4 —	0/4 —	0/4 —	4/4 (100%) x = 1.25	0/4 —

Results expressed as fraction positive, (%), and x: mean intensity of positivity (scale 1, 2, or 3). * "Epithelial cells" includes podocytes and parietal and tubular epithelium.

product). In lanes 5-8, the PCR products consist only of intense bands corresponding to G3PDH DNA (452 bp product), with no detectable band of HIV-1 envelope DNAs. The results were confirmed by Southern blotting (Fig. 1B).

DNA in situ PCR

Deletion of Klenow enzyme resulted in reduced sensitivity of the experiment in all groups (data not shown). Therefore, we adopted the use of 0.1 U/μl Klenow enzyme in our protocol. Negative control, the deletion of DNA Taq polymerase or DNase treatment, gave completely negative results.

The *in situ* PCR results for HIVAN and HIV-1 seropositive cases are itemized in Table 2. Among the Group 1 controls (24 HIV-1 seronegative cases), positive signal was observed in rare isolated interstitial leukocytes (7 cases; 29.2%) and tubular epithelial cells (4 cases; 16.7%). A few scattered exfoliated cells with apoptotic nuclei were identified as positive in the tubular lumen (12 cases: 50.0%). These exfoliated cells were identified as apoptotic based on their characteristic morphologic features including markedly condensed nucleus and cytoplasm. We also noted that apoptotic desquamated intratubular cells could readily be distinguished from true positive cells based on their deep red color, contrasting with the paler pink of true positive cells. By contrast, the 24 HIVAN specimens from Group 1 showed significantly increased positive signals in the nuclei of interstitial leukocytes (9 cases; 37.5%), focal positive signal in podocytes (8 cases; 33.3%), and rare positive signal in parietal cells (3 cases; 12.5%).
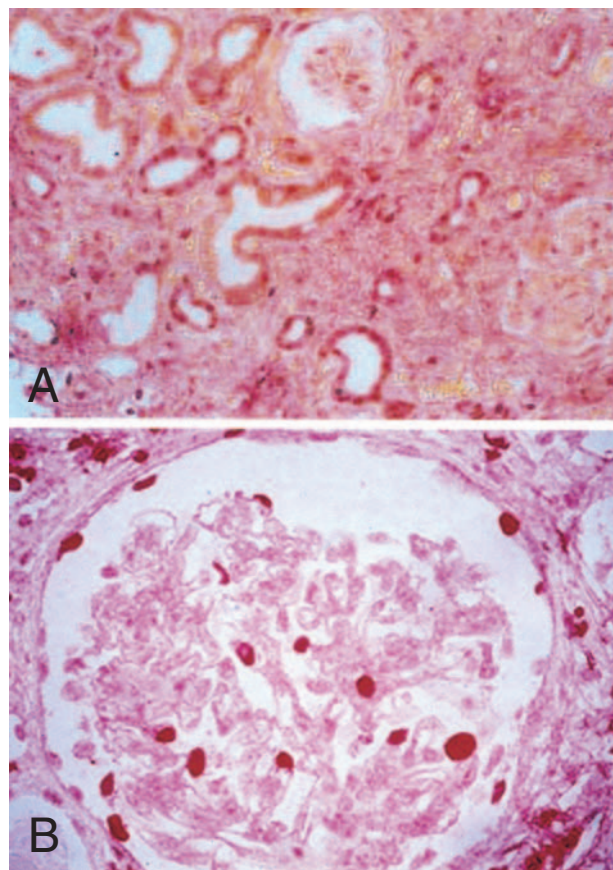
In the 5 autopsy specimens (Group 2), there was marked increase in the positivity of podocytes (3 cases; 60.0%), parietal cells (4 cases; 80.0%), renal tubular cells (5 cases; 100.0%), and interstitial leukocytes (5 cases; 100.0%) compared to Group 1 specimens. Although all specimens had some degree of autolysis, this did not interfere with the localization of positive signal to specific renal compartments.

In the 14 HIVAN cases fixed for 2 hrs (Group 3), positive signal was identified in the nuclei of podocytes (7 cases; 50.0%), parietal cells (6 cases; 42.9%), renal tubular cells (12 cases; 85.7%), and interstitial leukocytes (11 cases; 78.6%) (Fig. 2A,B). Apoptotic tubular cells, however, were also often detected as positive. Among the Group 3 controls (4 HIV-1 seronegative cases), the only positive signals were rare apoptotic tubular epithelial cells in the tubular lumina (Fig. 3).

Sensitivity and specificity of IS-PCR for detection of HIV DNA in renal tissue of HIVAN are presented in Table 3. Tubular intraluminal apoptotic cells were excluded from consideration. Sensitivity was 70.8% and specificity was 66.7% for biopsy specimens fixed with standard overnight fixation in 10% formalin (Group 1). Both the sensitivity and specificity were significantly improved (92.9% and 100% respectively) using brief 2 hr fixation in 4% paraformaldehyde (Group 3, p=0.016).

Table 3. Sensitivity and specificity of IS-PCR for detection of HIV DNA in HIVAN kidneys.

	SENSITIVITY	SPECIFICITY
Group 1 (biopsy specimens; overnight fixation in 10% formalin) (n = 48)	70.80%	66.70%
Group 2 (autopsy specimens; 24-72 hour fixation in 10% formalin or 4% paraformaldehyde) (n = 5)	100%	
Group 3 (biopsy specimens; 2 hour fixation in 4% paraformaldehyde) (n = 18)	92.90%	100%

Fig. 2. A. IS-PCR for HIV-1 envelope DNA performed in a case of HIVAN from Group 3 shows focal positivity as red-staining nuclei in the distribution of tubular epithelial cells and interstitial cells. Original magnification, x 200. **B.** IS-PCR on biopsy from a patient with HIVAN (Group 3) shows positivity in the nuclei of podocytes and parietal cells. Original magnification, x 400

Using amplification of ß-globin DNA, all cell types including renal epithelial cells, endothelial cells, mesangial cells, circulating nucleated cells in the vascular spaces, and infiltrating cells within the interstitium were clearly positive (Fig. 4). Transgenic mouse kidney, which was studied as a positive control, gave a positive signal in the majority of epithelial cells (data not shown).

DNA ISH

The signals observed using ISH showed a remarkably similar distribution as those observed using IS-PCR, but were slightly weaker in intensity. Figure 5A and 5B show the results of IS-PCR and ISH after IS-PCR, respectively, using serial sections from a specimen in Group 2. The same individual tubular epithelial cells

are stained by the two different techniques, confirming the specificity of the IS-PCR. Negative controls for ISH gave no detectable signal.

Double labeling

Using double labeling for CD3 and CD68, many of the interstitial cells that were positive for HIV-1 envelope DNA co-labeled with the pan-T cell marker (Fig. 6A), and fewer with the macrophage marker (Fig. 6B). Positive cells are recognized by their pink nuclei surrounded by a brown cell membrane. Negative controls in which primary antibody had been deleted were completely negative (data not shown).

Discussion

IS-PCR provides a major advance in the detection of foreign genes or gene alterations within tissue sections, allowing localization at the cellular level. To date,
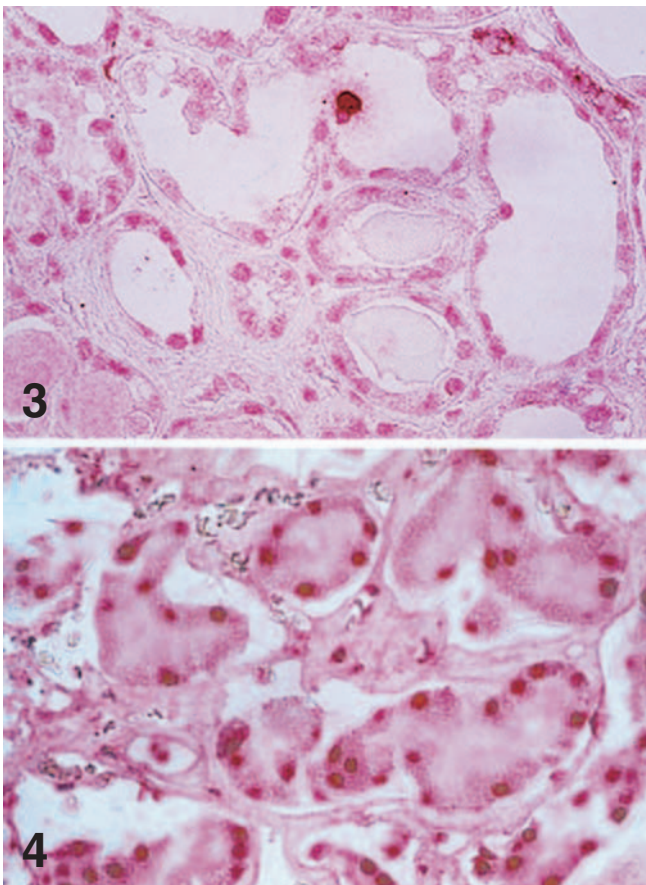
Fig. 3. S-PCR on an HIV seronegative control (Group 3) shows false positivity in an apoptotic tubular cell that has been shed into the tubular lumen. The apoptotic nucleus appears condensed and stains intensely red. Original magnification, x 400

Fig. 4. IS-PCR for b-globin DNA in a case of HIVAN from Group 3 shows diffuse and uniform positivity in the distribution of all tubular epithelial nuclei. Original magnification, x 400
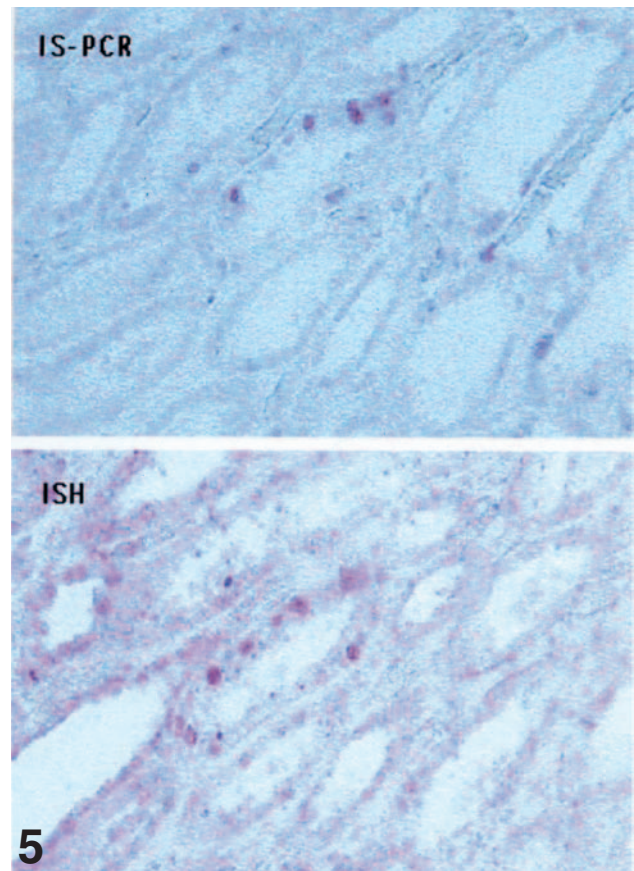
Fig. 5. A specimen from Group 2 shows serial sections stained for HIV-1 envelope DNA using IS-PCR alone **(top panel)** and IS-PCR followed by ISH **(bottom panel)**. Signal is identified in the same individual tubular epithelial cells with both techniques, confirming the specificity of the IS-PCR. Original magnification, x 400.
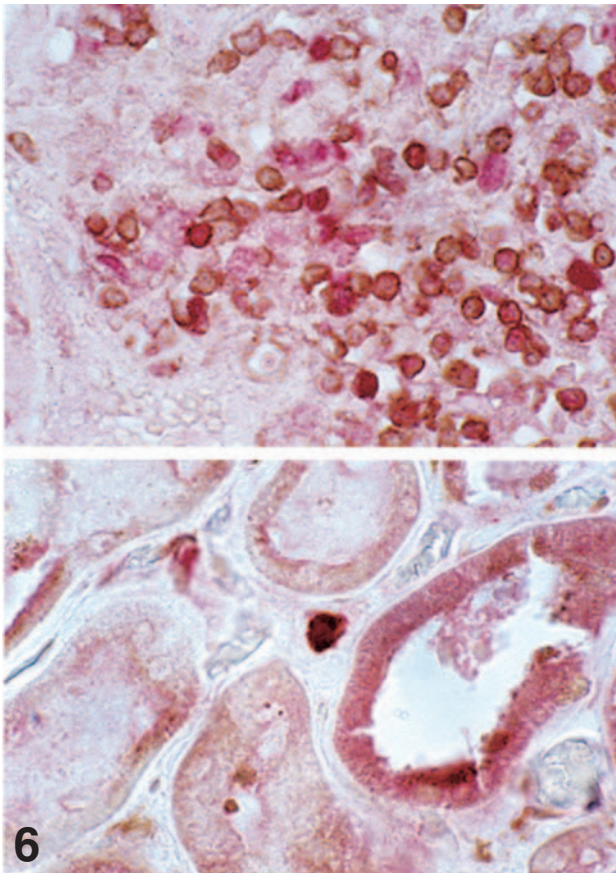
several studies have used this technique to demonstrate HIV sequences in the tissues of HIV-1 seropositive patients, including monocytes (Bagastra and Pomerantz, 1993; Patterson et al., 1993), lymph node (Embertson et al., 1993), cervix (Nuovo et al., 1993), placenta (Zavollus et al., 1994), and brain (Bagastra et al., 1996; An et al., 1999). This study builds on our previous demonstration of HIV-1 DNA in human renal tissue by IS-PCR (Bruggeman et al., 2000) and supports a role for direct viral infection in the pathogenesis of HIVAN.

In the application of IS-PCR to renal tissues, many technical problems had to be solved, including the method of fixation, protein digestion, amplification rate, prevention of diffusion of PCR products, and the specificity and sensitivity of the technique. Our study indicates that IS-PCR works inefficiently in archival biopsy tissues (Group 1), probably due to the poor quality of the nucleic acids and poor retention of PCR

Fig. 6. IS-PCR for HIV-1 envelope DNA followed by immunohistochemistry for pan-T cell marker shows double labeling of most of the interstitialleukocytes (red nucleus surrounded by brown nuclear membrane staining) **(top panel)**. Original magnification, x 400. IS-PCR for HIV-1 envelope DNA followed by immunohistochemistry for macrophage marker shows double labeling of an interstitial macrophage. No double labeling is identified in the adjacent tubular epithelial cells **(bottom panel)**. Original magnification, x 400.

products. Formalin is known to create cross-links between proteins and nucleotides. Prolonged or intense fixation may interfere with the amplification process due to extensive cross-linking of nucleotides within DNA, thereby altering or masking the target sequences. The use of microwaving may reduce the degree of cross-linking, but we found that microwave pretreatment gave a higher rate of false positivity. For example, using the seronegative controls from Group 1, we could identify increased numbers of interstitial cells and tubular cells as positive for HIV-1 by IS-PCR following microwave treatment. By contrast, in the positive control of transgenic mouse kidney with short and light fixation and no microwave digestion, many renal epithelial cells gave positive signal, as expected. Conditions were optimized using renal biopsy specimens subjected to a brief 2 hour fixation in 4% PFA (Group 3). HIV seropositive samples with brief fixation (Group 3) clearly showed numerous positive signals in the nuclei of renal tubular cells, podocytes, and parietal cells, even without microwave pretreatment. The seronegative controls were negative under the same conditions. Autopsy samples (Group 2) fixed for extended periods of time were abundantly positive, like the human biopsy samples with brief fixation (Group 3). While the specificity is unclear, these results indicate that the proper fixation time for IS-PCR is likely to be dependent in part on tissue size, with smaller specimens requiring shorter fixation.

Another important technical issue was the method of protein digestion. Although protein digestion is important to foster reagent penetration and target sequence amplification, we found this to be the most difficult variable to optimize. Over-digestion with proteinase K destroyed the tissue morphology and caused the diffusion of PCR products. Under-digestion, in contrast, prevented the PCR reaction from taking place due to poor permeability and excessive cross-linking. The 2 and 4 µg/ml solutions for the samples in Groups 3 and 2 respectively yielded optimal results.

For the amplification rate of HIV-1 envelope and ß-globin (data not shown) DNAs, we set the initial optimal conditions by conventional PCR. However, there are inherent differences between conventional PCR performed in solution and IS-PCR performed in tissue. The DNA strands that exist in fixed tissue are immobilized. Therefore, we found that prolonging the duration of each template melting cycle and reducing the number of cycles for *in situ* PCR, when compared to conventional PCR, gave the best results. In addition, because target sequences may be damaged or truncated during tissue processing and sectioning, we applied Klenow enzyme to restore the severed DNA. In particular, Klenow enzyme may reduce all 3' or 5' overhangs to blunt ends, which were abundant in apoptotic or necrotic DNA (Didenko et al., 2003). This step was critical to our protocol, as deletion of the Klenow step resulted in reduced sensitivity. Another critical variable was the diffusion of amplified PCR

products. Although this problem did not appear to affect the sensitivity of IS-PCR, it interfered with the efficacy of the ISH. To prevent the diffusion of PCR products, we applied biotin-14-dATP during IS-PCR prior to ISH, followed by gentle washing with solutions of appropriate stringency, and post-fixation with 4% PFA. The use of biotin-14-dATP gave markedly improved results, presumably because biotin is a large molecule that may help to immobilize the target sequence on binding. We did not use biotin-conjugated nucleotides and avidin-biotin complex (ABC) as the detection method to visualize the IS-PCR reaction results because of the report by An (1999) that the ABC technique proved less sensitive for the detection of HIV-1 DNA in brain. Instead, we chose digoxigenin-conjugated dUTP and alkaline phosphatase. In spite of the known intra-nuclear localization of endogenous alkaline phosphatase, various negative controls gave completely negative results with this protocol, indicating that the endogenous nuclear alkaline phosphatase did not pose a practical problem. Endogenous alkaline phosphatase is known to be inactivated by heat incubation and blocking with 0.02 M HCl (Ponder and Wilkinson, 1981; Kiyama and Emson, 1991). Therefore, we adopted a high temperature step for PCR and blocked with 0.02 M HCl to eliminate background.

Apoptotic cells were also detected as positive, although they could readily be distinguished from true positive cells by their morphologic features and deep red color. In contrast, true positive cells were paler pink. Preliminary experiments using terminal deoxynucleotide transferase-mediated dUTP nick end-labeling method on serial sections have confirmed that these deep red staining cells are indeed apoptotic (data not shown). We suspect that the fragmented DNAs generated during apoptosis may contain some closely related DNA sequences to the primers, allowing them to mis-anneal.

Based on the optimized results for Groups 2 and 3, our data demonstrate that renal epithelial cells are infected by HIV-1. The finding of HIV-1 infection consistently in specimens with HIVAN supports previous studies indicating the kidney is a reservoir for HIV-1 infection (Winston et al., 2001; Marras et al., 2002). Although *in vitro* studies have demonstrated the capacity of viral replication in renal endothelial or mesangial cells, but not visceral epithelial cells (Marras et al., 2002), our IS-PCR studies showed renal epithelial cells, including tubular cells, visceral epithelial cells, and parietal epithelial cells, as well as infiltrating leukocytes, are the major cell types infected in human kidney. These findings correlate with the reported phenotypic alterations in podocytes and tubular epithelial cells in human and murine HIVAN, which involve epithelial dedifferentiation and proliferation (Barisoni et al., 1999, 2000). The results of IS-PCR also correlate with the striking morphologic abnormalities typically observed in these cell types, including podocyte hypertrophy, hyperplasia, and apoptosis, and tubular epithelial degenerative changes and tubular microcyst formation

(D'Agati and Appel, 1997). HIV-1 has also been reported to induce apoptosis in the tubular epithelial cells of transgenic kidneys *ex vivo* (Bruggeman et al., 1997). *In vivo*, several HIV gene products such as Env, Tat, Nef and Vpr are known to promote cellular apoptotic signals (Schwartz and Klotman, 1998) in renal epithelial cells, and Env protein can induce apoptosis in infected CD4+ target cells (Laurent-Crawford et al., 1993). Therefore, our findings based on IS-PCR support that the characteristic morphologic abnormalities observed in HIVAN are the result of HIV-1 renal epithelial infection (Bruggeman et al., 2000; Marras et al., 2002).

In conclusion, IS-PCR is a powerful technique to detect and localize HIV-1 DNA in renal tissue sections. However, appropriate negative controls are essential to set the optimal conditions, including fixation time, protein digestion, and amplification rate. The specificity of the technique can be confirmed by use of IS-PCR followed by ISH using a probe internal to the IS-PCR products. In addition, immunohistochemistry or tissue counterstains performed following IS-PCR permit precise identification of the specific cell types infected. Our data further support the role of HIV renal parenchymal infection in the pathogenesis of HIVAN.

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