Renal Epithelium Is a Previously Unrecognized Site of HIV-1 Infection

Leslie A. Bruggeman, Michael D. Ross, Nozomu Tanji, Andrea Cara, Steven Dikman, Ronald E. Gordon, Godfrey C. Burns, Vivette D’Agati, Jonathan A. Winston, Mary E. Klotman, and Paul E. Klotman

Abstract. The striking emergence of an epidemic of HIV-related renal disease in patients with end-stage renal disease provided the rationale for the exploration of whether HIV-1 directly infects renal parenchymal cells. Renal glomerular and tubular epithelial cells contain HIV-1 mRNA and DNA, indicating infection by HIV-1. In addition, circularized viral DNA, a marker of recent nuclear import of full-length, reverse-transcribed RNA, was detected in the biopsies, suggesting active replication in renal tissue. Infiltrating infected leukocytes harbored more viral mRNA than renal epithelium. Identification of this novel reservoir suggests that effectively targeting the kidney with antiretrovirals may be critical for patients who are seropositive with renal disease. Thus, renal epithelium constitutes a unique and previously unrecognized cell target for HIV-1 infection.

Despite recent successes in therapy, an epidemic of renal disease related to HIV-1 infection has emerged, particularly in African Americans. HIV-associated nephropathy (HIVAN) is the third leading cause of end-stage renal disease (ESRD) in African Americans between the ages of 20 to 64, ranking only behind hypertension and diabetes (1) as a cause of ESRD. The pathogenesis of HIVAN is not clearly understood, but recent experimental evidence suggests a direct role for HIV-1 infection in renal pathogenesis (2–4).

In this article, we provide data that demonstrate the kidney to be a site of HIV-1 infection. We demonstrate here that renal epithelial cells are infected by HIV-1 in seropositive patients with renal disease. We used three methods to confirm the presence of HIV-1 in human kidney tissue: PCR for circularized episomal cDNA, mRNA in situ hybridization using two riboprobes that can detect spliced or full-length viral mRNA, and DNA in situ PCR. We use as a comparison infiltrating infected leukocytes from the same biopsy specimen in the in situ assays. In addition, we report evidence of viral replication in the kidneys of some patients with undetectable viral burdens, suggesting that the kidney may harbor replicating virus in ESRD patients.

Materials and Methods

Clinical Samples and Assays

Diseased human kidney tissue was obtained by biopsy on an Institutional Review Board-approved study from consenting patients who required diagnostic tissue evaluation (patients 1 to 21). Normal human kidney tissue (patients 23 and 24) and patient 22 also were obtained with consent on an approved study through a rapid autopsy program. Two independent pathologists, one blinded to patient identity and related clinical information, determined the renal diagnosis. Viral burden was determined from plasma using a quantitative reverse transcription-PCR method (AmpliCor assay, Roche Molecular Diagnostics, Indianapolis, IN). The lower limit of detection was 400 copies/ml for patients 1 to 19 and 50 copies/ml for patients 20 to 24. Patients who were classified as receiving highly active antiretroviral therapy were receiving a combination of one protease inhibitor and two nucleoside analogs.

PCR for Circularized Viral DNA

Circularized viral DNA was detected using a PCR technique as described (5,6). Unstained paraffin sections of biopsy material (5 μm) were scraped from slides into microcentrifuge tubes, and DNA was extracted using the QIAamp Tissue Kit (Qiagen, Valencia, CA). The sections were incubated in 180 μl of QIAamp tissue lysis buffer at 70°C for 5 min to melt the paraffin. The extraction was cooled to 50°C to begin the proteinase K digestion, and the DNA extraction was completed as described in the kit instructions with the following modifications. Kit AE buffer was replaced with 0.05X elution buffer adjusted to pH 9.0, and 0.5 μg of carrier tRNA was added per column. Eluate pH was then reduced to 7.0, and the samples were treated with RNase A (0.1 μg/ml for 30 min at 37°C) followed by lyophilization to reduce volume to approximately 50 μl. DNA extracted from the biopsies (25 μl of the above column eluate) was added to an amplification mixture including AmpliTaq buffer, 250 ng of each sense and antisense primers, 200 μM of each dNTP, and 1 μl AmpliTaq in a
total volume of 50 µl. The amplification used 45 cycles of melt at 94°C for 60 s, anneal at 60°C for 60 s, extend at 72°C for 15 s (with a 5-s addition per cycle). The run was completed with a 7-min extension at 72°C.

Aliquots of the PCR products were resolved on 2% agarose gels and Southern blotted to nylon membranes. The blot was hybridized with a 32P-labeled probe that was internal to the PCR primers corresponding to HXB2 LTR sequences (6). Hybridization was at 25°C in 6X SSC, 0.1% sodium dodecyl sulfate (SDS), 10X Denhardt’s solution for 1 h, followed by two washes in 6X SSC, 0.1% SDS at 25°C, and one wash at 42°C for 15 min. In addition, a PCR product for the circularized form was used as a probe under stringent conditions in Hybrisol (Intergen, Purchase, NY) at 42°C for 18 h, followed by stringent washes at 60°C in 0.1X SSC, 0.1% SDS. The remaining 25 µl of column eluate was used for control amplifications with the amplicer set for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA) using PCR conditions recommended by the manufacturer.

mRNA In Situ Hybridization

The in situ hybridization techniques were performed as described (2,7) with the following modifications. Human kidney tissue was obtained within minutes of biopsy and fixed for 2 h in phosphate-buffered saline (PBS) containing 4% formaldehyde (Polysciences Inc., Warrington, PA) at 4°C. After fixation, the tissue was manually processed for paraffin embedding, using 10-min incubations in graded ethanol, twice for 15 min in xylene and twice for 45 min in molten paraffin. Sections (5 µm) were cut, placed onto poly-L-lysine–coated slides, and dried for 2 h at 40°C. All cut sections and paraffin blocks were stored at −70°C until use. The in situ hybridization was performed as described using a digoxigenin-antidigoxigenin technique. Digoxigenin riboprobes were generated as described (2). The nef probe was from the plasmids pGM92 and pGM93 (NIH AIDS Research and Reference Reagent Program) and contain (in opposite orientations) 1.1 kb of the 3′ end of HXB2. The 1.1 kb riboprobes were randomly cleaved with alkali to an average length of less than 500 bp to facilitate tissue penetration. The gag probe was a 359-bp PCR fragment from HXB2 (nucleotides 1031 to 1390) subcloned into pGEM-T Easy (Promega, Madison, WI).

DNA In Situ PCR

Human kidney tissue was prepared as described for the in situ hybridizations and mounted on slides coated with 3-aminopropyltriethoxy silane. The sections (3 µm) were deparaffinized and rehydrated through a graded alcohol series. The sections were permeabilized with proteinase K (0.1 to 5.0 µg/ml) for 1 h at 37°C, followed by two washes in PBS, and allowed to air dry. The amplification was performed using OmniSlide (Hybaid, Middlesex, UK) in a reaction buffer of 1.5 mM MgCl2, 0.2 mM dNTP, 0.02 mM digoxigenin-11-dUTP (Roche), 1 ng/µl of each primer, and 0.05 U/µl Taq polymerase (Life Technologies, Gaithersburg, MD) in a total volume of 100 µl. The primers amplified nucleotides 6827 to 7367 of the env gene from NL4–3 (sense: 5′TGTCAGAAAGGTATCCTGAGCCAATTCC3′; antisense: 5′AGTAGAAGAATCTCCCTCACAATTA3′). The sections were covered with Frame-Seal Chambers (MJ Research, Watertown, MA) and preincubated at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 68°C for 30 s, extension at 72°C for 50 s, with a final extension at 72°C for 5 min. The sections were washed with graded SSC and incubated with 1% blocking reagent (Roche) for 30 min at room temperature. Detection of the digoxigenin label was with an alkaline phosphatase conjugated monoclonal antibody (Fab fragment, Roche) used at a dilution of 1:1000 for 1 h at room temperature. The sections were washed three times in PBS, and the alkaline phosphatase was visualized using SIGMA FAST (Sigma, St. Louis, MO).

Solution amplification using the DNA in situ PCR primers on normal human DNA did not produce any bands on agarose gel electrophoresis after 40 cycles of PCR (data not shown), indicating that the primers were not spuriously amplifying human genomic sequences. The primers used for the DNA in situ PCR were not contained within the probes used for the mRNA in situ hybridization, such that the two in situ procedures were detecting different sequences within the HIV-1 genome.

Results

Study Participants

The study group included seropositive patients with and without renal disease and a seronegative patient with renal disease (Table 1). Tissue from patients with renal disease was obtained by biopsy. Autopsy specimens provided normal renal tissue controls. Sixteen of the 21 seropositive patients with renal disease were diagnosed with HIVAN, with the remaining diagnoses consisting of diabetic nephropathy (patients 16 and 18), hypertensive nephropathy (patient 20), nephrosclerosis (patient 15), and allergic interstitial nephritis (patient 21). The majority of the patients were African American or Hispanic, and only one patient was Caucasian (tissue available only at autopsy). Most patients were receiving combination and highly active antiretroviral therapy. The clinical profiles of the participants in the study are summarized in Table 1.

Detection of Recent Infection by PCR

PCR amplification for HIV-1 DNA was performed on renal biopsy sections to detect the circularized, unintegrated form of viral DNA. This circularized form is a head-to-tail ligation of the long terminal repeats (2LTR form), which occurs after reverse transcription and nuclear import of the proviral DNA. Extrachromosomal circles have been shown previously to have a short half-life in replicating cells and are therefore considered a marker of recent viral infection and active replication (8). The PCR products were resolved on agarose gels (Figure 1a), and the identity of amplified DNA fragments was confirmed by Southern blotting using a 32P-end-labeled oligonucleotide that was internal to the PCR primers (Figure 1b). A control amplification for G3PDH was performed to confirm that sufficient DNA was extracted from each biopsy for a successful amplification (Figure 1c).

Sixteen of the 23 HIV-1 seropositive patients contained the 2LTR circularized DNA, indicating recent infection of cells residing in the kidney (summary in Table 1). Included in the renal disease group were four patients (patients 6, 15, 19, and 21) who had undetectable viral burden as measured by circulating levels of virus. In three of the four patients, virus replication persisted in the kidney, even during periods of successful therapy. In addition, the seropositive patients without renal disease had evidence of viral replication in the kidney. Because the PCR was performed on whole biopsy tissue, the observed viral replication may reflect either replication in...
Table 1. Participant clinical profiles at the time of renal biopsy or autopsy and summary of in situ and PCR analyses

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender/Age (Yr)</th>
<th>Racial/Ethnic Identity</th>
<th>Antiretroviral Treatment</th>
<th>CD4 Count (Cells/mm³)</th>
<th>Viral Burden (Copies/ml)</th>
<th>Circularized Virus PCR</th>
<th>mRNA In Situ Hybridization</th>
<th>In Situ DNA PCR</th>
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<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>−</td>
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<td>+</td>
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<td>+</td>
<td>nd</td>
<td>f,g,h</td>
</tr>
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</table>

*HAART, highly active antiretroviral therapy; ddI, dideoxyinosine; NA, not applicable; nd, not done.

b Not done at the time of renal biopsy or autopsy.

c HIV-1 seronegative patient with lupus nephritis.

d Low limit of detection was 50 copies/ml.

f Failed HAART.

g Tissue was from autopsy, and mRNA degradation may have occurred.

h Tissue was from autopsy and had significant autolysis; identity of positive cells could not be determined accurately.

Intrinsic renal cells or the presence of infected leukocytes trapped in the vascular space or infiltrating the interstitium.

In several patients with high viral burdens, we were unable to detect circularized HIV-1 DNA in kidney by PCR. In one patient (patient 11), for example, more than $10^5$ copies/ml of virus were detected in peripheral blood, but no circularized virus was detected in kidney. The lack of detectable replication in these patients may reflect technical problems in the PCR study. First, the focal distribution of the infected cells (see below) would lend to sampling errors. Second, the sequences for the PCR primers and hybridization probes were taken from the cloned virus HXB2. These PCR primers were selected from known conserved regions of the LTR but may not be of sufficient sequence homology to detect all strains present in the study group, especially if the patients were infected with non-clade B strains. This varied degree of homology can be seen in the discrepancies between the amount of amplified products and the intensity of the Southern hybridization signal. For example, in patients 7, 9, 16, 18, and 20, amplified bands of the correct size were observed but either did not hybridize or hybridized weakly (Figure 1, a and b, lanes 7, 9, 16, 18, and 20). This indicated high sequence similarity with the PCR primers but not with the internal hybridization probe. Alternatively, patients 5 and 21 had very faint amplified products on the gel that hybridized strongly on the blot (Figure 1, a and b, lanes 5 and 21), suggesting a low copy number of 2LTR circles but good sequence homology to the internal probe.

Detection of Viral mRNA by In Situ Hybridization

The detection of virus by PCR in patients with renal disease led us to examine whether renal parenchymal cells could be infected by HIV-1 in vivo. Because this PCR study could not identify the cells infected in the biopsy, mRNA in situ hybridizations using two different riboprobes were performed to determine which cells were expressing viral mRNA. Figure 2 shows representative mRNA in situ hybridizations for both infected and uninfected
patients. There was no hybridization signal in the HIV-1 seronegative patient (Figure 2, a and b). In HIV-1 seropositive patients, tubular epithelial cells were the primary cell type expressing HIV-1 mRNA (Figure 2, c and d). Figure 2d shows four tubules containing cells that expressed viral mRNA. In biopsies with tubules expressing HIV-1 mRNA, there were frequently other tubules in the same section that were negative, suggesting focal areas of infection. A typical pathologic feature of HIVAN is the microcystic dilation of tubules with the presence of both cellular and proteinaceous casts. We and others have previously reported that renal epithelial cells undergo apoptosis in response to HIV-1 gene expression (2,3,9,10). This causes the cells to lose contact with their basement membrane and slough into the lumen of the tubule. Cellular casts were observed within the lumens of infected tubules (Figure 2d), and frequently these cellular casts were also positive for viral mRNA. The proteinaceous casts, without cells, did not demonstrate a positive hybridization signal.

The cellular distribution of the viral mRNA was observed primarily in the cytoplasm of the infected cells as shown in Figure 2, e and f. Because of the typical basal position of the nucleus in the tubular cells, the most intense staining appeared in the cytoplasm above the nucleus on the apical side (Figure 2e). Cells with apical or centrally positioned nuclei also had cytoplasmic staining, but the most intense staining was on the basal or lateral side of the cell, respectively (Figure 2f). In many of the infected cells, there also seemed to be fainter staining in the nucleus, which may reflect nascent transcripts. Uninfected cells did not have any background level of staining (Figure 2f).

The level of viral mRNA expression in the renal cells could be compared to infected lymphocytes in several patients with severe tubulointerstitial disease. In these patients, the interstitium was heavily infiltrated with leukocytes forming microabscesses (Figure 3a). In these abscesses, the leukocytes exhibited levels of expression that exceeded the levels observed in the neighboring infected renal epithelial cells. As described above, the most intense staining in both the infiltrates and the epithelial cells was in the cytoplasm (Figure 3, b and c). The cytoplasmic staining of the infiltrates was very intense as compared with the cytoplasmic staining of the tubular epithelial cells; however, the low level of nuclear staining seemed similar in both cell types.

By in situ hybridization using both nef and gag riboprobes, 14 of 20 renal biopsies from HIV-1 seropositive patients with renal disease had epithelial cells infected by HIV-1 (summary in Table 1). In the positive biopsies, interstitial infiltrating leukocytes frequently expressed HIV-1 mRNA. These cells serve as a useful comparison in a cell type known to support productive infection. The expression of viral mRNA in renal cells was qualitatively less than infected leukocytes in the same patient but still easily detected. Infected epithelial cells also appeared in cellular casts. Viral RNA also was detected in biopsies from patients with undetectable viral burdens, suggesting that the kidney may be a reservoir for HIV-1 infection. Patient 21 had an undetectable viral burden at the time of biopsy (<50 copies/ml) but had tubular epithelial cells actively expressing viral mRNA (using both nef and gag riboprobes). The detection of gag and nef viral mRNA suggests that there is not a block in the export of full-length transcripts in renal epithelial cells, as has been shown to occur in HIV-1 infection of the brain (11,12).

Detection of Viral DNA by In Situ PCR

To confirm the mRNA in situ hybridization studies, some of the biopsies were analyzed by in situ DNA PCR, which would allow detection of viral DNA in similarly fixed sections. Figure 4 shows a representative DNA in situ PCR from patients with and without renal disease. A seropositive patient without renal...
Figure 2. *In situ* hybridization for HIV-1 mRNA in renal biopsies. (a and b) Renal biopsy from seronegative patient 13 with renal disease demonstrating no hybridization in the sense control (a) or the antisense (b) hybridization of a serial section. (c and d) Renal biopsy from seropositive patient 19 with renal disease. No hybridization was observed in the sense control (c). Antisense hybridization in a serial section (d) shows positive hybridization as purple/brown staining in the cytoplasm in tubular epithelial cells. Tubular lumens (TL) were frequently filled with cellular casts (CC) or protein casts (PC). The CC resulted from apoptosis of infected epithelial cells that slough into the lumen; note that viral RNA also was detected in the CC but not in PC. (e) A photographic enlargement of the boxed region in d indicates cytoplasmic staining of viral mRNA. The nucleus of the epithelial cell typically is positioned on the basal side of the cell and the cytoplasmic staining (arrowheads) thus appears more on the apical surface. (f) *In situ* hybridization of renal biopsy from patient 11 showing epithelial cell expression of viral mRNA. Note that the position of the cytoplasmic staining (arrowheads) is opposite the location of the nucleus; cells with apical nuclei have basal staining, cells with basal nuclei have apical staining, and some cells have lateral staining. The faint staining of the nuclei of positive cells probably reflects nascent transcripts, because no staining was observed in the nucleus of a negative cell (arrow). Magnifications: 125× in b; 60× in d; 200× in f.
disease (Figure 4a) had rare interstitial cells that were positive, presumably infiltrating leukocytes (Figure 4b). A patient with HIVAN, shown in Figure 4c, demonstrates the typical features of the disease with microcystic tubular distortion, interstitial fibrosis, immune cell infiltrates, and collapsing focal glomerulosclerosis. In these specimens, viral DNA was detected in the nucleus of tubular and glomerular epithelial cells, as well as in interstitial cells (Figure 4, c and d). Both the type of infected cells and the focal distribution were similar to results obtained using mRNA in situ hybridization. Although the number of patients without renal disease analyzed was small, there did not seem to be any renal parenchymal cell infection in the absence of renal disease.

In both the mRNA in situ hybridization and the DNA in situ PCR, epithelial cells within the glomerulus were also positive (Figure 5). Parietal epithelial cells lining Bowman’s capsule were positive in some patients, as well as cells within the glomerulus in a distribution consistent with visceral epithelial cells (podocytes). The parietal epithelial cells typically have very thin, flat cytoplasmic compartments, making the cytoplasmic staining of in situ hybridizations difficult to observe (Figure 5a), but the DNA in situ hybridization clearly showed the presence of nuclear viral DNA (Figure 5b). The podocytes are located throughout the glomerular tuft and were also positive for both viral DNA and RNA (Figure 5).

Discussion

Accumulating evidence supports the hypothesis that renal epithelial cells can be infected by HIV-1. In 1989, Cohen, et al. (13) reported a DNA in situ hybridization that was positive in a tubule in a single patient. Other groups, however, failed to support that finding (4,14). In 1993, Kimmel et al. (15) detected viral DNA in microdissected glomeruli and tubules using a PCR-based technique. These data could not exclude contaminating leukocytes and did not detect mRNA. In vitro data have also been conflicting. Renal epithelial cells can be infected in vitro using human primary cell lines and non–laboratory-adapted viral isolates (P.E. Klotman unpublished data; 3,16). Because p24 production in these studies is low, the question remains whether this represents only an in vitro phenomenon or one that also occurs in vivo. HIV-1 infection of epithelial cells has been documented in other organs. For example, cervical mucosa (17–21) and intestinal epithelia (22–24) can be productively infected in vitro using non–CD4-dependent routes of entry. Again, whether these findings are applicable to the in vivo condition in humans is unknown.

The data we present here indicate that HIV-1 gains entry into epithelium and that full-length mRNA are generated. The mechanism for entry in renal cells remains unknown. Recently, Conaldi et al. (3) demonstrated that low levels of HIV infection could be detected in renal epithelial cells grown in vitro, and they found that up to 25% of the cells express CD4 as well as one of the major HIV-1 co-receptors, CXCR4, by reverse transcription-PCR. Whether CD4 is expressed on the cell surface in sufficient amounts to make this a plausible entry mechanism in vivo remains to be determined. Furthermore, the renal expression of co-receptors in humans remains uncharacterized. Alpers and co-workers (25,26) recently demonstrated that CCR5 is not expressed in normal or rejecting human kidneys. Similarly, they showed that CCR5 is not expressed in normal, SHIV-infected, HIV-2–infected, and SIV-infected Macaque kidneys. Thus, a role for CCR5 in renal cell infection seems unlikely, although entry through a CD4-dependent mechanism in concert with an alternative co-receptor remains plausible.
Our studies also indicated that ESRD patients had evidence of active replication in the kidney even during periods of successful therapy, suggesting that the kidney may function as a reservoir for replicating virus. There is increasing evidence for viral reservoirs, either as latently infected cells or as cells with continued low levels of replication, such as quiescent memory T cells, lymphoid tissues, and semen (27–34). The ability of the virus to persist after aggressive therapy may result from either a true latency or sequestration in an anatomic site not effectively exposed to drug (35,36).

Although our studies showed that within the kidney both renal epithelial cells and interstitial leukocytes were infected by HIV-1, the replication detected in our PCR assay could not discern between the two compartments. The data raise the possibility, however, that the kidney represents another protected compartment that is difficult to access with current therapeutic strategies, similar to the central nervous system (35,36).

In summary, HIV-1 infects renal epithelial cells, and both RNA (\textit{nef} and \textit{gag}) and DNA (\textit{env}) can be detected in renal cells even in patients with undetectable levels of viral RNA in peripheral blood. Active replication, as indicated by the presence of extrachromosomal circular DNA, whether in leukocytes or renal epithelial cells, persisted in patients with undetectable viral burdens, which suggests that the kidney harbors replicating virus. Thus, the kidney may be an anatomic site that

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\textbf{Figure 4.} DNA \textit{in situ} PCR for HIV-1 proviral DNA. (a) \textit{In situ} PCR on seropositive patient 23, who had no renal disease. Positive amplification signal for viral DNA is seen as red staining in the nucleus. Magnification, $\times$ 60. (b) Photographic enlargement of boxed region in a, showing rare positive cells within the interstitium (arrowheads), likely infiltrating leukocytes. (c) \textit{In situ} PCR on seropositive patient 20 with HIV-associated nephropathy. HIV-1 DNA was detected in the nucleus of tubular epithelial cells, interstitial cells, and also cells lining Bowman’s capsule and within the glomerulus. Magnification, $\times$ 60. (d) Photographic enlargement of boxed region in c, showing positive tubular epithelial cells (arrowheads) as well as cells within the interstitium (arrowheads). Within some tubules, positive nuclei (arrowhead) were adjacent to negative nuclei (arrows).
sequesters infected leukocytes, as well as a newly appreciated cellular site of infection and/or a possible site of replication. In addition, infection of renal epithelial cells may have a direct role in initiating pathogenic processes in HIVAN, but the mechanisms of pathogenesis are not addressed by these studies.

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References


Figure 5. Detection of infected glomerular epithelial cells by mRNA in situ hybridization and DNA in situ PCR. (a) Antisense in situ hybridization in a glomerulus showing both parietal epithelial cells (arrows) and podocytes (arrowheads) expressing viral mRNA. The cytoplasm (arrows) of the parietal epithelial cell is very thin along Bowman’s capsule and showed positive hybridization for viral mRNA. The nucleus (asterisk) showed faint staining. Viral mRNA expression was also observed in podocytes (arrowheads), which are distributed throughout the glomerulus but are also present at the extreme edges of the glomerular tuft on the outside of capillary loops. (b) DNA in situ PCR in a glomerulus demonstrating the presence of viral DNA in the same cells detected by in situ hybridization; the parietal epithelial cells (arrows) and the podocytes (arrowhead). G, glomerulus; US, urinary space. Magnifications: 200× in a and b.


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