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Chemokine Receptor CCR5 and CXCR4 Expression in HIV-Associated Kidney Disease

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Abstract. The chemokine receptors CCR5 and CXCR4 have been identified as essential coreceptors for entry of HIV-1 strains into susceptible cells. Direct infection of renal parenchymal cells has been implicated in the pathogenesis of HIV-associated renal disease, although data are conflicting. The localization of CCR5 and CXCR4 in kidneys with HIV-associated renal disease is unknown. Formalin-fixed, paraffin-embedded renal biopsies from patients with HIV-associated nephropathy (HIVAN) ($n = 13$), HIV-associated immune complex glomerulonephritis ($n = 3$), HIV-associated thrombotic microangiopathy ($n = 1$), and HIV-negative patients with collapsing glomerulopathy ($n = 8$) were analyzed in this study. Cellular sites of expression of CCR5 and CXCR4 were identified by immunohistochemistry and by *in situ* hybridization. The presence of HIV-1 was detected by immunohistochemistry and by *in situ* hybridization. Expression of both chemokine receptors CCR5 and CXCR4 was undetectable in intrinsic glomerular, tubular, and renovascular cells in all analyzed cases. In the presence of tubulointerstitial inflammation, CCR5 and CXCR4 expression was localized to infiltrating mononuclear leukocytes. HIV-1 protein was undetectable by immunohistochemistry in all cases of HIV-associated renal disease. HIV-1 RNA was identified in one case of HIVAN but was restricted to infiltrating leukocytes. HIV-1 RNA was not detected in intrinsic renal cells in all analyzed cases. Identifying the cellular expression of HIV-coreceptors CCR5 and CXCR4 may help to clarify which tissues are permissive for direct HIV infection. These data do not support a role of productive HIV-1 infection of renal parenchymal cells in the pathogenesis of HIV-associated renal disease.

Renal disease may complicate approximately 10% of patients infected with HIV, with a striking predominance in African-Americans (1–3). Several clinically and morphologically diverse renal syndromes have been described in HIV-infected patients (4,5). The syndrome most frequently reported in renal biopsy series from HIV-infected individuals has been termed HIV-associated nephropathy (HIVAN) (5,6). Characteristic pathologic features of HIVAN include a collapsing form of focal and segmental glomerulosclerosis with hypertrophy and hyperplasia of glomerular visceral epithelial cells, extensive tubulointerstitial injury, including microcystic tubular dilation, and prominent endothelial cell tubuloreticular inclusions (5,6). Clinically, such patients frequently show nephrotic-range proteinuria with renal insufficiency and rapid progression to end-stage renal disease (2,4). Other lesions reported in renal biopsies from HIV-infected patients are immune complex-mediated renal diseases, including membranoproliferative glomerulonephritis, membranous glomerulonephritis, and IgA nephropathy (5,7–9). Renal thrombotic microangiopathy has been increasingly reported in HIV-infected humans (reviewed in references (10) and (11). Tubulointerstitial inflammation is present to variable degrees in all varieties of HIV-associated renal disease (12–14).

The pathogenesis of HIV-associated renal disease is poorly understood (2,3). Proposed mechanisms include: (*1*) direct HIV infection of renal parenchymal cells (*e.g.*, visceral epithelial cells, tubular epithelial cells, and renovascular endothelial cells); (*2*) indirect injury to the kidney by renal cellular uptake of circulating virally encoded molecules; or (*3*) indirect injury to the kidney through release of cytokines by infected mononuclear cells in the circulation or infiltrating the kidney (2,3). Furthermore, different mechanisms might be involved in the pathogenesis of the different variants of HIV-associated renal disease. Circulating immune complex deposition and *in situ* mechanisms of immune-mediated renal disease may underlie the pathogenesis of glomerulonephritis in HIV-infected patients (7). In addition, there may be a role for hepatitis C coinfection in some cases of HIV-associated immune complex

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glomerulonephritis (8). Some renal disease in HIV-infected individuals, however, may represent chance occurrence unrelated to HIV infection itself.

New directions in AIDS research of viral entry, tropism, and pathogenesis were initiated by the recent discovery that members of the chemokine receptor family act as necessary coreceptors together with CD4 for entry of HIV-1 into susceptible cells (15). HIV-1 strains previously characterized as T lymphocyte-tropic have been shown to bind to the chemokine receptor CXCR4 as a condition of entry into mammalian cells, while macrophage-tropic strains of HIV-1 require the chemokine receptor CCR5. Expression of these molecules appears to play a key role in determining which tissues are permissive for direct HIV infection.

Expression of the HIV coreceptors CCR5 and CXCR4 in human kidneys with features of HIV-associated renal disease is completely unknown. In previous studies from our laboratories, expression of both chemokine receptors CCR5 and CXCR4 was not identified by either immunohistochemical (16) or *in situ* hybridization (17,18) techniques in intrinsic renal parenchymal cells in normal human kidneys, in renal allograft nephrectomies, and in human renal biopsies with a variety of different glomerular and interstitial diseases. However, *in vitro* studies show that the expression of certain chemokine receptors in mononuclear cells is regulated by a number of stimuli (reviewed in references (19) and (20). An upregulation of CCR5 or CXCR4 expression in intrinsic renal cells might therefore require specific cytokine stimulation present in the course of HIV infection.

In this study, we tested the hypothesis that the relevant chemokine coreceptors for HIV infection not constitutively expressed in normal kidney may be upregulated in the setting of HIV infection and thereby permit infection of renal tissue. We assessed the cellular sites of expression of chemokine receptor CCR5 by immunohistochemistry and *in situ* hybridization, and CXCR4 by *in situ* hybridization, in renal biopsies obtained from HIV-infected patients with features of HIVAN, HIV-associated immune complex glomerulonephritis, and HIV-associated thrombotic microangiopathy. Additional tissues examined within this study consisted of human renal biopsy material with features of a collapsing glomerulopathy from patients without clinical evidence of HIV infection. Furthermore, we provide corresponding data for the presence of HIV-1 protein by immunohistochemistry and for the presence of HIV-1 RNA by *in situ* hybridization in each of these cases.

Materials and Methods

Source of Tissue

Formalin-fixed, paraffin-embedded renal biopsy tissue specimens obtained between 1984 and 1998 at the George Washington University Medical Center (Washington, DC), the New York University (New York, NY), and the University of Washington (Seattle, WA) were included in this study. Several cases had been included in previous studies investigating HIV-associated renal disease (7,8,14,21–23). All renal biopsy cases with sufficient tissue for complete immunohistochemical and *in situ* hybridization evaluation after completion of diagnostic workup and previous investigations were included. Renal biopsies from 13 patients with HIVAN were studied. All cases demonstrated variable degrees of a collapsing form of focal and segmental glomerulosclerosis with microcystic tubular dilation. Additionally, renal biopsies from three patients with HIV-associated immune complex glomerulonephritis were analyzed, including two cases of diffuse proliferative glomerulonephritis and one case of membranous nephropathy. One renal biopsy with HIV-associated renal thrombotic microangiopathy was also examined. For HIV-negative disease controls, we analyzed kidney samples from eight patients with collapsing glomerulopathy and negative HIV serology. Tissue sections contained 1 to 20 glomeruli (mean, 4 glomeruli) from patients with HIVAN and 2, 4, and 10 glomeruli, respectively, from patients with HIV-associated glomerulonephritis, and 13 glomeruli from the patient with HIV-associated thrombotic microangiopathy. Kidney samples from HIV-negative patients with collapsing glomerulopathy included 2 to 12 glomeruli (mean, 5 glomeruli). The demography of the patient populations in New York and Washington, DC has been published previously (21,24).

Formalin-fixed, paraffin-embedded HIV-1-infected human peripheral blood mononuclear cell (PBMC) pellets were generated as controls for immunohistochemical detection of HIV-1 p24 antigen and for *in situ* hybridization of HIV-1 RNA. Human PBMC were obtained from HIV antibody-negative volunteer donors as described previously (25). Briefly, the PBMC layer was removed after centrifugation of whole blood, washed, and resuspended in RPMI 1640 containing 16% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (0.3 mg/ml). The mononuclear cells were stimulated with phytohemagglutinin (5 μg/ml) and 5% nonrecombinant human interleukin-2 (Pharmacia, Piscataway, NJ) as described (25). Forty-eight hours after stimulation, the human PBMC were infected with a cell-free solution of HIV-1 strain LAI. A full-length clone of HIV-1_{LAI} [pBRU3] was obtained from Dr. Michael Emerman, Fred Hutchinson Cancer Research Center, Seattle, WA (26). Cell-free stock solutions of HIV-1_{LAI} were generated by transfection of this plasmid into 293T cells.) Cells were harvested 48 h after infection and counted in a hemocytometer. Several different preparations were generated by mixing defined concentrations of HIV-1-infected PBMC with defined concentrations of uninfected PBMC. The different HIV-1-infected/uninfected PBMC preparations contained HIV-1-infected PBMC in concentrations ranging from 5 to 50%. These mixed PBMC samples were subsequently centrifuged, and the remaining cell pellet was fixed in 10% phosphate-buffered formalin and embedded in paraffin using standard protocols for tissue preparation. Four-micrometer-thick sections were generated as controls for immunohistochemical and *in situ* hybridization procedures.

Antibodies

CCR5. A murine monoclonal antibody MC5 directed against human chemokine receptor CCR5 has previously been characterized for specificity by Western blotting and fluorescence-activated cell sorter analysis (16), and was found suitable for the specific detection of CCR5 in formalin-fixed, paraffin-embedded tissue sections after heat-mediated antigen retrieval procedures (16).

HIV-1. A murine monoclonal antibody p24, clone Kal-1, directed against an epitope of the core protein p24 of HIV-1 was purchased from DAKO (Carpinteria, CA). Specificity of this antibody for the detection of HIV-1 p24 has been demonstrated previously by immunoprecipitation, Western blotting, and immunohistochemistry (27), and it has been demonstrated previously to recognize HIV-1 p24 in formalin-fixed, paraffin-embedded tissue sections (27,28).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections according to protocols that we have used previously (29). Four-micrometer sections of tissue samples were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Sections that were subsequently incubated with the anti-CCR5 antibody were pretreated by steam heating for 20 min in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA), according to the instructions of the manufacturer. Nonspecific binding was blocked by incubation in 10% normal horse serum (Vector). The sections were then incubated for 1 h at room temperature with the primary antibody diluted in phosphate-buffered saline plus 1% bovine serum albumin (Sigma, St. Louis, MO). After washes in phosphate-buffered saline, the sections were incubated with biotinylated horse anti-mouse antibody (Vector). A Tyramide Signal Amplification (TSA™-Indirect, NEN™ Life Science Products, Boston, MA) was performed according to the manufacturer's instructions. Finally, 3,3′ diaminobenzidine (with nickel chloride enhancement) was used as a chromogen. Sections were counterstained with methyl green, dehydrated, and coverslipped. The complete biopsies were examined, and the number of CCR5 protein-expressing cells per glomerular cross-section was calculated in each case. Negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with isotype-matched, irrelevant murine monoclonal antibodies (DAKO).

Molecular Probes

CCR5. A 1.1-kb sequence of DNA coding for human CCR5 was subcloned into pcDNAI/amp (Invitrogen, San Diego, CA) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, and NIH, originally provided by Dr. Nathaniel Landau) and then linearized with *Hin*dIII and transcribed with Sp6 for the antisense probe or linearized with *Sph*I and transcribed with T7 for the sense probe. Sensitivity and specificity of the CCR5 antisense riboprobe has been demonstrated previously by Northern analysis and by *in situ* hybridization (17).

CXCR4. A 1.1-kb sequence of DNA coding for human CXCR4 was subcloned into pcDNAI/amp (Invitrogen) (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, and NIH, originally provided by Dr. Nathaniel Landau) and then linearized with *Hin*dIII and transcribed with Sp6 for the antisense probe or linearized with *Xba*I and transcribed with T7 for the sense probe. Sensitivity and specificity of the CXCR4 antisense riboprobe have been demonstrated previously by Northern analysis and by *in situ* hybridization (18).

HIV-1. Four DNA templates that collectively represent 90% of the HIV-1 genome were purchased from Lofstrand Labs (Gaithersburg, MD): *gag*, *Sac*I-*Bgl*II, 1.4 kb; *gag/pol*, *Bgl*II-*Eco*RI, 2.6 kb; *pol/vif/vpr/rev/tat/vpu*, *Kpn*I-*Kpn*I, 2.2 kb; *env/vpr/rev/tat/vpu*, *Eco*RI-*Bam*HI, 2.7 kb. Antisense and sense riboprobes were generated from all templates using Sp6 or T7 according to the manufacturer's instructions. Radioactivity was introduced by synthesis of RNA using ^{35}S-UTP. The generated probes were combined to yield one sense or antisense probe cocktail. Specific detection of HIV-1 RNA in formalin-fixed, paraffin-embedded tissue sections by *in situ* hybridization with an estimated sensitivity of 30 to 300 copies of target RNA has been described previously by numerous groups using identical RNA probes (30–35).

In Situ Hybridization

HIV-1 RNA, CCR5 mRNA, and CXCR4 mRNA were detected in tissue sections using *in situ* hybridization techniques according to protocols used previously (29). Riboprobes for *in situ* hybridization were generated from cDNA using ^{35}S-UTP. Four-micrometer sections of formalin-fixed, paraffin-embedded tissue samples were deparaffinized and rehydrated through xylene and graded ethanols, washed with 0.5× SSC (Life Technologies, Grand Island, NY), and digested with proteinase K (5 μg/ml; Sigma). Sections that were subsequently hybridized with HIV-1-specific riboprobes were analyzed in duplicate.

In one section, the *in situ* hybridization was performed according to the following protocol. The second section was additionally pretreated by steam heating for 20 min in Antigen Unmasking Solution (Vector) before the hybridization, according to the manufacturer's instructions. Prehybridization was performed for 2 h by adding 100 μl of prehybridization buffer (0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM ethylenediaminetetra-acetic acid, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol). The hybridizations were started by adding 500,000 cpm of ^{35}S-labeled riboprobe in 50 μl of prehybridization buffer and allowed to proceed overnight at 50°C. After hybridization, sections were treated with RNase A (20 μg/ml; Sigma), followed by three high-stringency washes in 0.1× SSC/0.5% Tween 20 (Sigma) for 40 min each at 50°C, and several 2× SSC washes. After the tissue was dehydrated and air-dried, it was dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 2 wk (HIV-1) or 6 wk (CCR5, CXCR4), respectively. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated, and coverslipped. Positive cellular labeling was defined as five or more silver grains concentrated over a single cell on the slides hybridized with the antisense probe, and little or no signal present on the sense control slides. The complete biopsies were examined, and the numbers of CCR5 mRNA and CXCR4 mRNA expressing cells per glomerular cross-section were calculated in each case. Positive controls for the detection of CCR5 and CXCR4 mRNA consisted of several allograft nephrectomy specimens with features of severe rejection, as published previously (17,18).

Results

CCR5 Expression in HIV-Associated Renal Disease

By both immunohistochemistry and *in situ* hybridization, CCR5 expression was absent in intrinsic glomerular cells in all cases of HIVAN, HIV-associated glomerulonephritis, HIV-associated thrombotic microangiopathy, and HIV-negative collapsing glomerulopathy. Parietal epithelial cells, visceral epithelial cells, mesangial cells, and glomerular endothelial cells in glomeruli, with and without features of collapse, did not express detectable CCR5 (Figure 1, A through D). A few CCR5-positive cells were detected in the capillary lumina of some glomeruli in cases of all different groups (Figure 1, A and B, Table 1). These cells likely represented circulating mononuclear leukocytes.

All cases of the different disease groups analyzed in this study showed variable degrees of tubulointerstitial inflammation ranging from very mild, focal, perivascular infiltrates of leukocytes to severe, diffuse interstitial mononuclear cell infiltrates. CCR5-expressing cells were demonstrable by both immunohistochemistry (Figure 1, E and F) and *in situ* hybridization (Figure 1, G and H) in these infiltrates in the tubulo-

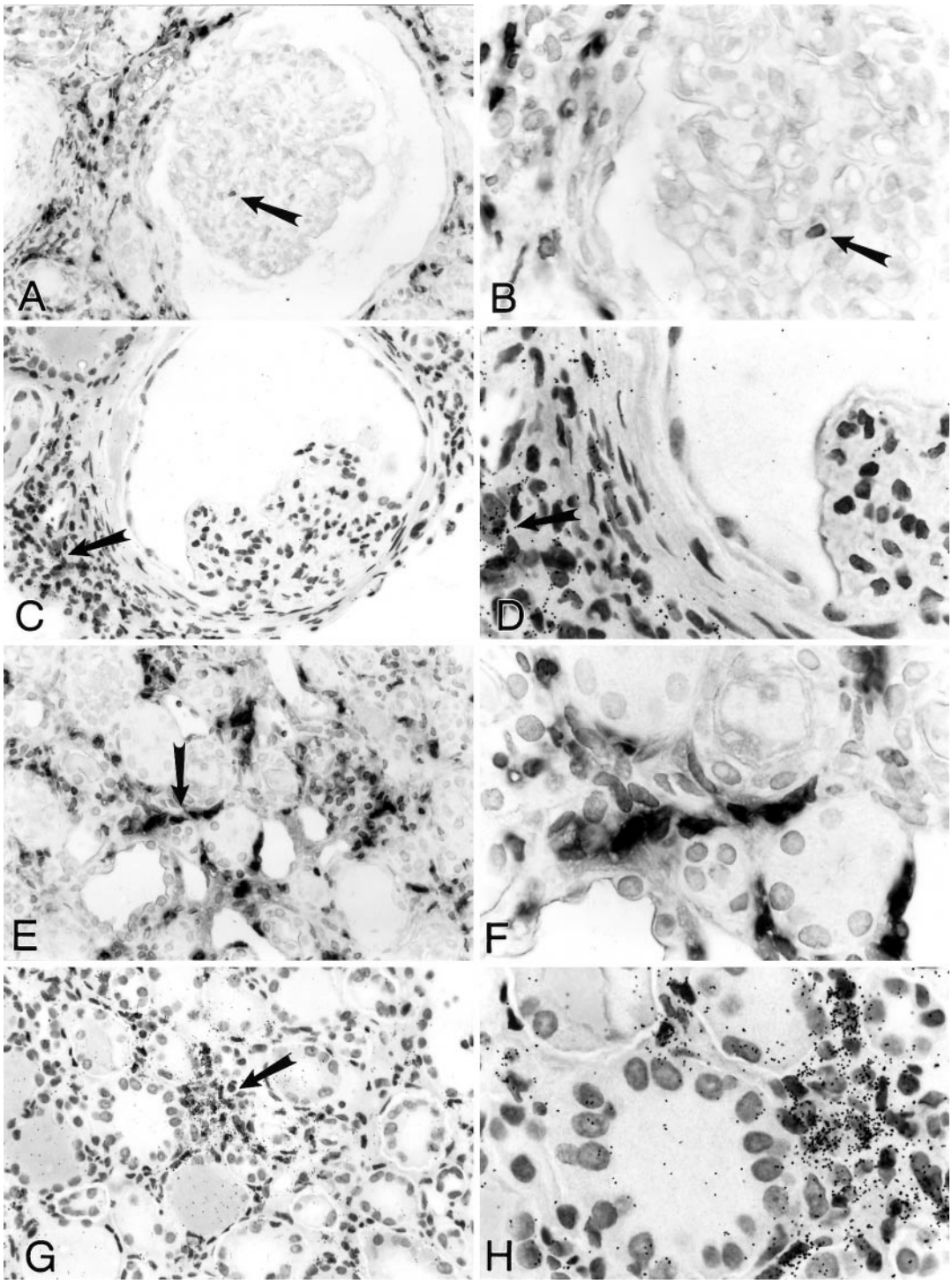

Figure 1. Chemokine receptor CCR5 expression in HIV-associated nephropathy (HIVAN). (A and B) Low and high magnification illustration of CCR5 immunohistochemistry. Several CCR5 protein-expressing leukocytes (black) were demonstrable at sites of periglomerular leukocytic infiltration. A single CCR5 protein-expressing circulating mononuclear leukocyte (arrow) is demonstrable within a glomerular capillary lumen. CCR5 protein expression was absent in intrinsic glomerular cells. (C) No CCR5 mRNA expression was seen in intrinsic glomerular cells by *in situ* hybridization. (D) High-power view of Panel C shows CCR5 mRNA (black silver grains) restricted to mononuclear cells within a periglomerular inflammatory cell infiltrate (arrow). (E) In the tubulointerstitial compartment, CCR5 protein-expressing infiltrating mononuclear cells (black) were demonstrable by immunohistochemistry at sites of leukocytic infiltration. The area around CCR5-expressing cells in Panel E (arrow) is illustrated at higher magnification in Panel F. CCR5 protein expression was absent in tubular epithelium. (G) *In situ* hybridization for CCR5 mRNA matched the results of the CCR5 immunohistochemistry. The area around CCR5 mRNA-expressing cells in Panel G (arrow) is illustrated at higher magnification in Panel H. Numerous CCR5 mRNA-expressing leukocytes (black silver grains) were detectable at a site of tubulointerstitial infiltration; no specific hybridization signal for CCR5 mRNA was present in tubular epithelial cells. Methyl green counterstain in A, B, E, and F; hematoxylin and eosin counterstain in C, D, G, and H. Magnification: ×400 in A, C, E, and G; ×1000 in B, D, F and H.

Table 1. Chemokine receptor-expressing cells within glomeruli[a]

Category	CXCR4 mRNA-Positive Cells/Glomeruli	CCR5 Protein-Positive Cells/Glomeruli	CCR5 mRNA-Positive Cells/Glomeruli
HIV-associated nephropathy ($n = 13$)	0.4 ± 0.6	0.3 ± 0.3	0.1 ± 0.1
HIV-associated immune complex glomerulonephritis ($n = 3$)	1.4 ± 1.7	0.3 ± 0.3	0.2 ± 0.4
HIV-associated thrombotic microangiopathy ($n = 1$)	4.0	1.1	0.1
Collapsing glomerulopathy, HIV-negative ($n = 8$)	0.6 ± 0.4	0.3 ± 0.3	0.1 ± 0.3

[a] Data are given as mean values ± SD of chemokine receptor-expressing cells per glomerular cross-sections.

interstitial compartment of all analyzed cases. The expression of CCR5 was restricted to infiltrating mononuclear cells (Figure 1, E through H). The number of CCR5-expressing cells correlated with the degree of tubulointerstitial mononuclear cell infiltration. No difference in the CCR5 expression pattern on infiltrating leukocytes was seen between patients with HIV-associated renal disease or HIV-negative disease controls (data not shown). Tubular epithelial cells, whether located in areas with absence of interstitial inflammation, at sites of severe tubulointerstitial mononuclear cell infiltration, or at sites of microcystic tubular dilation, showed no detectable CCR5 expression (Figure 1, E through H). CCR5 expression was completely absent in endothelial cells and smooth muscle cells of the vascular compartment.

The number of leukocytes exhibiting expression of the CCR5 protein as detected by immunohistochemistry correlated with the number of leukocytes synthesizing detectable CCR5 mRNA in all individual cases (Figure 1, compare Panels F and H). However, in the glomerular compartment slightly more CCR5-positive leukocytes were identified by immunohistochemistry compared with *in situ* hybridization (Table 1).

CXCR4 Expression in HIV-Associated Renal Disease

By *in situ* hybridization, CXCR4 mRNA expression remained undetectable in cells clearly identifiable as renal parenchymal cells of the glomerular, tubular, and vascular compartments in all analyzed cases. At sites of collapsing glomeruli, CXCR4 mRNA expression was typically absent (Figure 2, A and B). However, within all analyzed cases small numbers of individual CXCR4 mRNA-expressing cells were identified within glomeruli (Figure 2, C and D) (Table 1). We were unable to clearly determine whether these CXCR4 mRNA-expressing cells were intrinsic glomerular cells or circulating leukocytes, although we favor the latter interpretation. All of the biopsy tissue was consumed during the course of the present studies, and so material was unavailable for double-labeling studies that might confirm this interpretation.

Within the tubulointerstitial compartment, CXCR4 mRNA expression was restricted to infiltrating mononuclear leukocytes at sites of tubulointerstitial inflammation (Figure 2, E and F). There was no difference in the number of detectable CXCR4 mRNA-expressing leukocytes between HIV-1-positive or -negative renal tissues. CXCR4 mRNA expression was undetectable in tubular epithelial cells, including those at sites of tubulointerstitial inflammation and at sites of microcystic tubular dilation (Figure 2, E and F). Renovascular endothelial cells did not show detectable CXCR4 mRNA expression.

Comparison of CCR5 and CXCR4 expression in serial sections of the analyzed specimens revealed that both chemokine receptors were expressed in a large percentage of interstitial infiltrating mononuclear leukocytes. The number of CCR5- or CXCR4-expressing cells, respectively, correlated positively with the degree of tubulointerstitial inflammation. No obvious differences in the distribution pattern of CCR5- or CXCR4-expressing infiltrating leukocytes were evident. Because of the technical limitation of serial sections, we could not properly address the question of whether CCR5 and CXCR4 were coexpressed in the same subset of infiltrating mononuclear leukocytes, or whether CCR5 and CXCR4 were expressed in different leukocyte subsets. As indicated above, exhaustion of the biopsy samples precluded double-labeling studies that would address this issue.

Specificity of the HIV-1 p24 Immunohistochemistry and of the HIV-1 RNA in Situ Hybridization

Immunohistochemical detection of HIV-1 proteins in formalin-fixed, paraffin-embedded renal tissue sections has been very unreliable and frequently resulted in nonspecific immunostaining in tissue obtained from HIV-negative controls (36,37). We therefore performed a series of experiments to control the specificity of our procedures. As positive controls, formalin-fixed, paraffin-embedded PBMC pellets, containing HIV-1-infected PBMC, reproducibly demonstrated positive immunostaining (Figure 3, A through C). Furthermore, the number of cells with positive immunohistochemical signal within the pellet closely reflected the number of HIV-1-infected PBMC mixed in the pellet preparation (Figure 3, A through D). No immunostaining was detectable in HIV-1-infected PBMC when the primary antibody was substituted by an irrelevant, isotype-matched mouse IgG preparation (data not shown). PBMC pellets that contained no HIV-1-infected cells

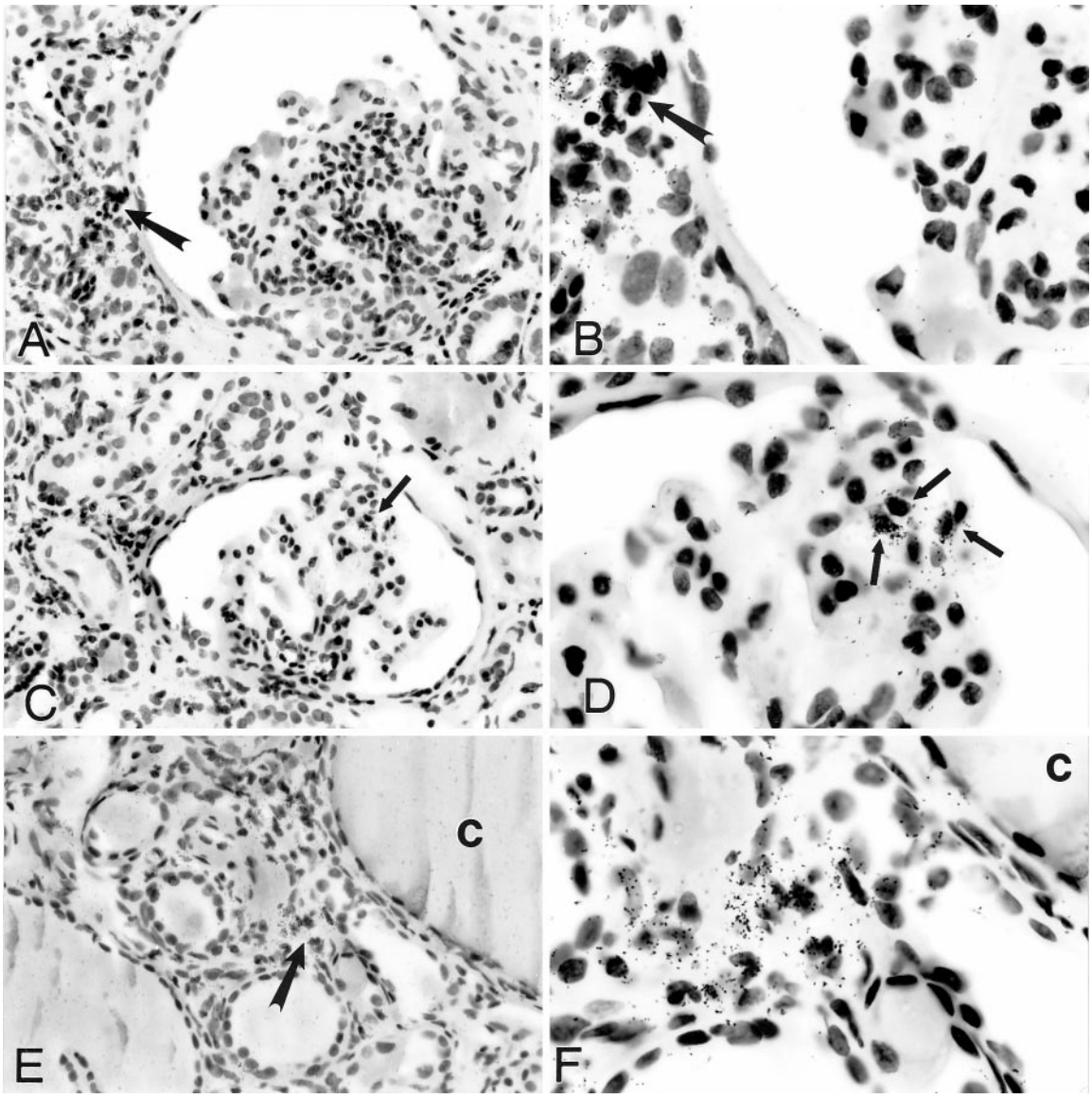

Figure 2. Chemokine receptor CXCR4 expression in HIVAN. (A and B) Low and high magnification illustration of CXCR4 *in situ* hybridization. CXCR4 mRNA expression was absent in a glomerulus with typical features of a collapsing glomerulopathy. Several mononuclear leukocytes within a periglomerular interstitial infiltrate (arrow) showed detectable CXCR4 mRNA expression (black silver grains). (C and D) Low and high magnification illustration of CXCR4 *in situ* hybridization. CXCR4 mRNA expression (black silver grains) was localized to a cluster of three individual cells (arrows) whose precise identity cannot be ascertained by their location or appearance in these sections, within the illustrated glomerular cross-section. (E and F) Low and high magnification illustration of *in situ* hybridization for CXCR4 mRNA. Within the tubulointerstitial compartment, CXCR4 mRNA expression (black silver grains) was clearly restricted to inflammatory interstitial mononuclear leukocytes (arrow), whereas tubular epithelial cells did not demonstrate detectable CXCR4 mRNA expression. CXCR4 mRNA expression was absent at sites of microcystic tubular dilation (c). Hematoxylin and eosin counterstain in A through F. Magnification: ×400 in A, C, and E; ×1000 in B, D, and F.

showed no detectable immunostaining with the HIV-1 p24 antibody (Figure 3D). Renal biopsy sections from patients with collapsing glomerulopathy and negative HIV serology ($n = 8$) consistently showed absent immunohistochemical staining when incubated with the HIV-1 p24 antibody (data not shown).

PBMC pellets containing HIV-1-infected PBMC consistently showed detectable *in situ* hybridization signal for HIV-1 RNA (Figure 3E). The number of PBMC with positive hybridization signal closely reflected the number of HIV-1-infected PBMC mixed in the pellet preparation. No difference in the number of HIV-1 RNA-positive PBMC was seen in the *in situ* hybridization procedures with and without heat-mediated pretreatment of the PBMC pellet specimens before hybridization (data not shown). No hybridization was seen when identical procedures were performed with substitution of a control sense probe for hybridization (Figure 3F). All renal biopsies obtained from HIV-negative control patients showed absent hybridization signal for HIV-1 RNA (data not shown).

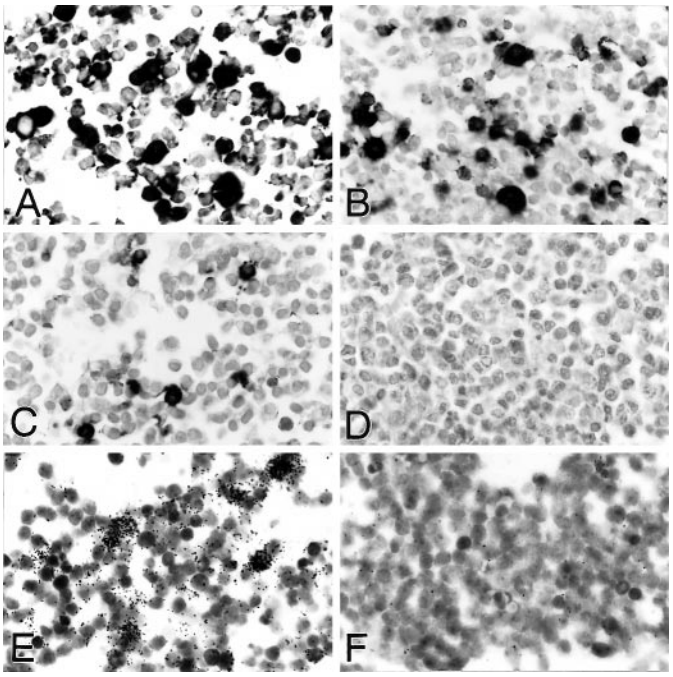

Figure 3. Specificity of the HIV-1 p24 immunohistochemistry (A through D) and HIV-1 RNA *in situ* hybridization (E through F). Formalin-fixed, paraffin-embedded peripheral blood mononuclear cell (PBMC) pellet preparations containing defined concentrations of HIV-1-infected PBMC were used. (A) Positive immunostaining for HIV-1 p24 (black) was detectable in approximately 50% of the cells in a section of a formalin-fixed, paraffin-embedded PBMC pellet containing 50% HIV-1-infected PBMC. (B) Immunohistochemistry for HIV-1 p24 labels approximately 33% of the PBMC in a cell pellet containing 33% HIV-1-infected PBMC. (C) Approximately 5% of the PBMC are immunohistochemically labeled in a cell pellet containing 5% HIV-1-infected PBMC. (D) PBMC pellets that contained no HIV-1-infected cells showed no detectable immunostaining with the HIV-1 p24 antibody. (E) *In situ* hybridization detects HIV-1 RNA (black silver grains) in numerous HIV-1-infected cells in a section of a formalin-fixed, paraffin-embedded PBMC pellet containing 33% HIV-1-infected PBMC. (F) No hybridization signal was seen in the same PBMC pellet as illustrated in Panel E when identical procedures were performed with substitution of a control sense probe for hybridization. Methyl green counterstain in A through D; hematoxylin and eosin counterstain in E and F. Magnification: ×1000.

Detection of HIV-1 in Biopsies from Patients with HIV-Associated Renal Disease

By immunohistochemistry, HIV-1 p24 protein was not detected in glomerular (Figure 4B), tubulointerstitial (Figure 4E), or vascular compartments of all cases of HIVAN ($n = 13$), HIV-associated glomerulonephritis ($n = 3$), and HIV-associated thrombotic microangiopathy ($n = 1$).

In situ hybridization detected no HIV-1 RNA in glomerular (Figure 4, C and D), tubular (Figure 4F), or vascular cells in all analyzed cases. However, in one case of HIVAN, HIV-1 RNA was detectable within one small lymphoid follicle located in the tubulointerstitium (Figure 4, G and H). HIV-1 RNA was clearly restricted to infiltrating mononuclear leukocytes.

Discussion

Renal disease is frequently reported in a large percentage of HIV-infected humans, although the mechanisms leading to HIV-associated renal injury are largely unknown. Direct infection of renal parenchymal cells by HIV in susceptible patients has been proposed (2,3). The results of the present study do not support a role for productive infection of renal cells by HIV in the pathogenesis of HIV-associated renal disease. The two principal HIV coreceptors, the chemokine receptors CCR5 and CXCR4 that mediate entry of HIV-1 strains into susceptible cells, were not expressed by intrinsic renal cells in HIV-associated renal disease, but were demonstrable in circulating and infiltrating leukocytes at sites of tubulointerstitial inflammation. Furthermore, using sensitive immunohistochemical and *in situ* hybridization techniques, HIV-1 protein and RNA were undetectable in renal parenchymal cells in biopsies with features of HIV-associated renal disease. Small numbers of HIV-1 RNA-expressing mononuclear leukocytes were demonstrable in the tubulointerstitium in one case of HIVAN.

Major insights into the mechanisms of HIV infection have been obtained from the discovery that members of the chemokine receptor family act as necessary coreceptors, together with CD4, for entry of AIDS viruses into mammalian cells (reviewed in reference (15). Chemokine receptors represent a family of structurally and functionally related seven transmembrane-spanning, G protein-coupled receptors (19,20,38). Recent observations indicate that certain chemokine receptors may also facilitate infection by immunodeficiency viruses in a CD4-independent manner (39,40). By facilitating entry into cells, these receptors determine viral tropism. CXCR4 is a coreceptor for strains of HIV-1 that infect T lymphocyte cell lines (T-tropic strains), and CCR5 serves as a coreceptor for HIV-1 isolates that infect macrophages and activated T lymphocytes (M-tropic strains) (15). Additional *in vivo* observations support the central role of CCR5 and CXCR4 in the pathogenesis of HIV infection in humans. Individuals homozygous for a mutant allele of the CCR5 gene bearing a 32-nucleotide deletion (CCR5 delta 32), which leads to an inactive variant of CCR5, have been linked to protection from HIV-1 infection (41,42). The consequence of the heterozygous state is not clear, but it may delay the progression to AIDS in infected individuals (41–43). Additionally, individuals homozygous for a mutation of the only identified CXCR4 ligand, stromal cell-derived factor-1, appear to have some degree of protection against disease progression after HIV infection (44).

The expression of chemokine receptors is not limited to leukocytes and lymphoid tissues. Tissue-specific expression of the HIV coreceptors CCR5 and CXCR4 most likely play a role in determining whether tissues are permissive for direct HIV infection *in vivo*. CCR5 mRNA was detectable by Northern blotting in RNA isolated from small intestine, ovary, and lung (45), and CCR5 protein was detectable by immunohistochemistry in the central nervous system (on neurons, astrocytes, and microglia) and on endothelium, vascular smooth muscle cells, and fibroblasts in several analyzed parenchymal tissues (46). Two recent studies by members of our group investigated the

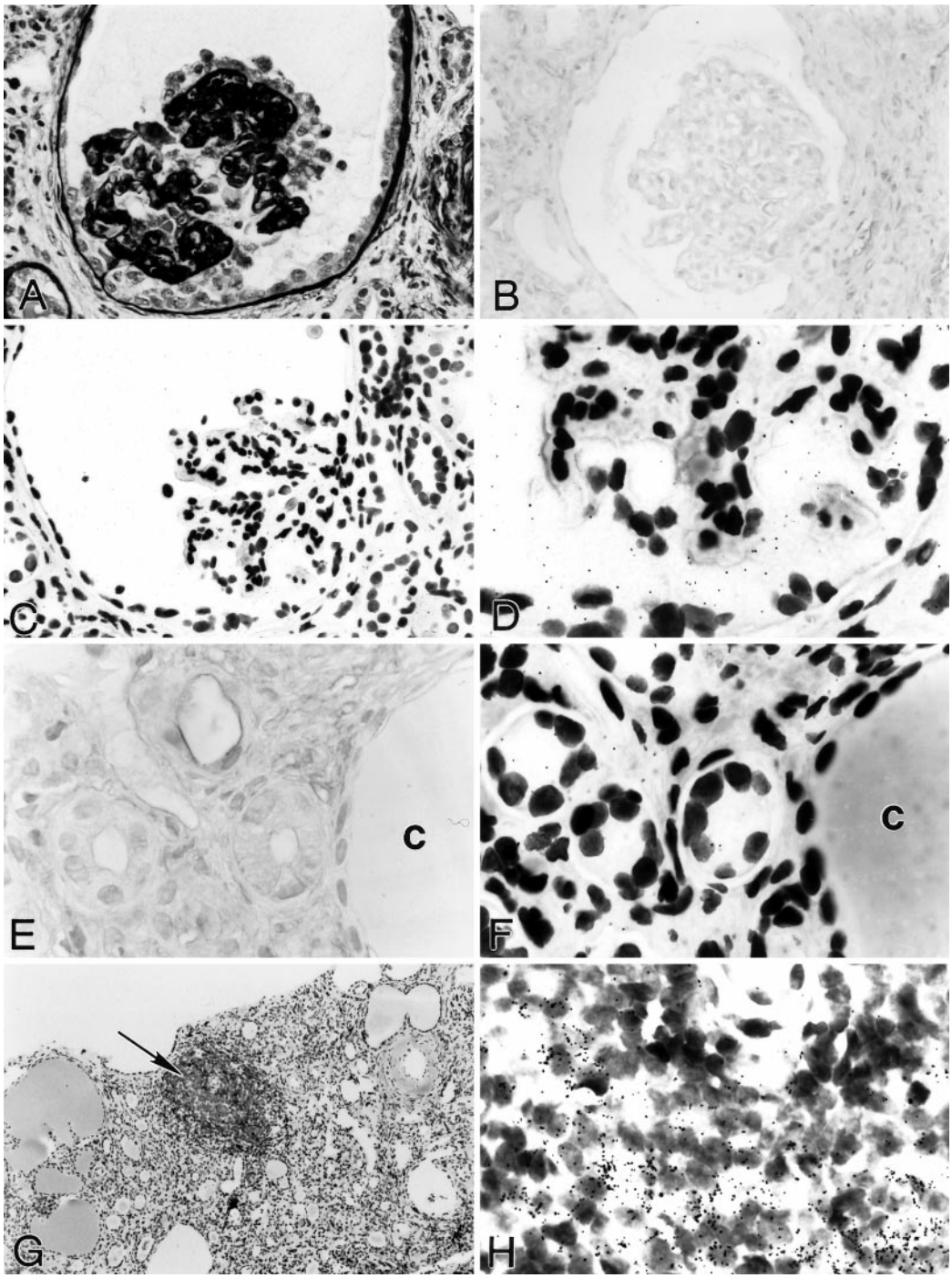

Figure 4. Detection of HIV-1 protein and HIV-1 RNA in HIVAN. (A) Characteristic pathologic features of HIVAN, including global glomerular collapse, glomerulosclerosis, and prominent hypertrophy/hyperplasia of epithelial cells at the outer aspect of the tuft. (B) By immunohistochemistry, HIV-1 p24 protein remained undetectable at sites of glomerular lesions. (C and D) Low and high magnification illustration of *in situ* hybridization for HIV-1 RNA. No specific hybridization signal for HIV-1 RNA was seen at sites of glomerular lesions. Nonspecific background hybridization signal was seen in the urinary space. (E and F) Within the tubulointerstitial compartment, HIV-1 p24 protein (E) and HIV-1 RNA (F) were undetectable in tubular cells of all analyzed cases with HIV-associated renal disease by either immunohistochemistry (E) or *in situ* hybridization (F). c, microcystic tubular dilation. (G and H) HIV-1 RNA was identified at a site of extensive tubulointerstitial inflammation in one case of HIVAN. (G) *In situ* hybridization after heat pretreatment detected HIV-1 RNA localized to infiltrating mononuclear leukocytes, which are focally organized as a lymphoid follicle. A portion of the follicle, indicated by the arrow, is illustrated at higher magnification in Panel H. Positive hybridization signal is visualized by black silver grains. HIV-1 p24 protein was undetectable by immunohistochemistry in this lymphoid follicle (not shown). Silver methenamine staining in A; methyl green counterstain in B and E; hematoxylin and eosin counterstain in C, D, and F through H. The architecture of the glomerulus and the pathologic features of HIVAN were visualized best with the silver methenamine staining. This staining is not suitable as counterstain for either immunohistochemical or *in situ* hybridization procedures. Magnification: ×400 in A through C; ×1000 in D through F and H; ×100 in G.

expression of CCR5 in normal kidneys, kidneys with various glomerular diseases, kidneys with interstitial diseases, and rejected allograft nephrectomies (16,17). By either *in situ* hybridization (17) or immunohistochemistry (16), CCR5 was undetectable in intrinsic renal cell types of the glomerular, tubular, or vascular compartments. The expression of CCR5 was restricted to infiltrating mononuclear leukocytes at sites of tubulointerstitial and vascular injury. The total number of CCR5-expressing cells correlated with the number of CD3-positive T lymphocytes at sites of tubulointerstitial inflammation (16).

T lymphocytes, monocytes, and neutrophils express the chemokine receptor CXCR4 (47), however, CXCR4 is by far the most widely expressed of the functional chemokine receptors in nonhematopoietic cells (38). High transcript levels have been demonstrated in several tissues, including heart, brain, liver, and colon (48). We have recently reported that CXCR4 mRNA expression is absent in intrinsic glomerular, tubular, and renovascular cells in native normal kidneys and in rejected allograft nephrectomies (18). In the presence of renal interstitial inflammation, CXCR4 expression was localized to a large fraction of infiltrating leukocytes (18).

The present study does not provide evidence for an upregulated renal parenchymal expression of the HIV coreceptors CCR5 or CXCR4 in kidneys with features of HIV-associated renal disease. Expression of the two analyzed chemokine receptors was clearly limited to infiltrating leukocytes at sites of tubulointerstitial inflammation. Individual CCR5- and CXCR4-expressing cells were identified within glomeruli. Those cells likely represented circulating leukocytes. Because of the complete exhaustion of the biopsy material, a combination of *in situ* hybridization for chemokine receptor mRNA with immunohistochemical labeling of cellular phenotypes as described previously (18) was impossible. However, a previous detailed immunohistochemical analysis of the immune cell populations present in HIV-associated kidney disease (14) quantified the numbers of macrophages and T lymphocytes in glomeruli obtained from patients with HIVAN and with HIV-associated immune complex glomerulonephritis. In that study (14), the total number of leukocytes per glomerular cross-section was 2.7 ± 1.1 (mean $\pm$ SEM) in HIVAN and 5.5 ± 2.8 in HIV-associated immune complex glomerulonephritis. The numbers of chemokine receptor CCR5- and CXCR4-expressing cells in glomeruli, identified in the present study (as detailed in Table 1), correlate well with the previously published number of circulating leukocytes in HIV-associated renal disease.

Chemoattraction of CCR5- and CXCR4-expressing mononuclear leukocytes to sites of tubulointerstitial inflammation is not specific for HIV-associated renal disease. Similar numbers of CCR5- or CXCR4-expressing leukocytes were detected in HIV-negative collapsing glomerulopathy (present study) and in HIV-negative patients with a variety of different glomerular and interstitial diseases (16–18).

We have previously investigated whether the major HIV receptor CD4 is expressed by intrinsic renal parenchymal cells. Unpublished studies by our group of immunohistochemically detectable expression in human renal tissues and published studies of cultured human mesangial cells (49) have failed to demonstrate detectable expression of CD4 on any cells except infiltrating leukocytes in kidney tissues. A single report, published as a letter but without further corroboration in the literature, has asserted that CD4 can be expressed by human mesangial cells (50).

Previous investigations that were aimed to detect HIV-1 proteins or genomic material in human renal biopsies from HIV-infected individuals revealed conflicting data. Cohen *et al.* demonstrated the presence of HIV-1 protein in the cytoplasm of tubular epithelium and in a few glomerular visceral epithelial cells in human biopsies with HIVAN by immunohistochemistry and of HIV-1 genomic material within a small number of tubular epithelial cells and within both glomerular parietal epithelial and visceral epithelial cells by *in situ* hybridization (51). Kimmel *et al.* detected HIV-1 protein by immunofluorescence in acid-treated kidney sections obtained from patients with HIV-associated immune complex glomerulonephritis (7) and HIV-associated IgA nephropathy (9). Others, however, have not been able to replicate these results (52,53). Furthermore, Nadasdy *et al.* and a recent study by Yamamoto *et al.* testing several different HIV-1 antibody preparations detected positive immunostaining not only in renal biopsies with features of HIVAN but also in uninfected control tissues, raising serious concerns regarding the specificity of immunohistochemical staining with some HIV-1 antibodies (36,37). In a previous study, two of the authors of the present study were able to demonstrate HIV-1 DNA in microdissected glomeruli, tubules, interstitial cells, and inflammatory infiltrating cells from renal biopsies of HIV-infected patients using PCR amplification techniques (22). Identification of this genomic material did not correspond to manifestations of HIV-associated renal disease, as HIV genome was equally detectable in renal tissues of HIV-infected patients with and without clinical or pathologic evidence of renal disease (22). Although PCR amplification is likely to be the most sensitive technique for the detection of HIV genomic material, the difficult microdissection procedures entail a risk of contamination by circulating or infiltrating leukocytes, and therefore there remains some uncertainty about whether infection of renal parenchymal cells has been conclusively demonstrated in this study. Our study, using less sensitive techniques, cannot exclude the possibility of latent infection of tissues or the possibility of low-level replication with virus copy numbers below the threshold for detection by either immunohistochemistry or *in situ* hybridization.

A second part of this present study therefore readdressed the still conflicting question of whether productive HIV-1 infection (detection of HIV-1 protein or RNA) occurs in renal parenchymal cells in HIV-associated renal disease *in vivo*. We used highly sensitive immunohistochemistry and *in situ* hybridization techniques that identified productive HIV-1 infection in lymphoid and neuronal tissues (30–35,54) and that have not reportedly been used for studies in kidney tissues. Immunohistochemical detection of HIV-1 was performed with a well-characterized, commercially available murine monoclonal

antibody (DAKO, anti-p24, clone Kal-1) (27,28) according to the recently published protocol of Strappe *et al.* (54). *In situ* hybridization utilized radiolabeled RNA probes generated from commercially available cDNA templates of the HIV-1 genome (30) according to the initial protocol of Fox *et al.* with some modifications to achieve a higher sensitivity. These modifications included pretreatment of the formalin-fixed, paraffin-embedded tissue sections with proteinase K and heat-mediated procedures as described (34).

Both immunohistochemistry and *in situ* hybridization demonstrated excellent specificity in PBMC control pellets and uninfected control tissues and were sensitive enough to easily detect productively infected leukocytes in the PBMC pellet preparations. However, both HIV-1 p24 antigen and HIV-1 RNA remained completely undetectable in renal parenchymal cells in all analyzed cases of HIV-associated renal disease. In one single renal biopsy with HIVAN, HIV-1 RNA was localized to mononuclear cells at a site of extensive tubulointerstitial inflammation.

Our data do not provide evidence for productive HIV-1 infection of renal parenchymal cells in the pathogenesis of HIV-associated renal disease. We recognize that our methods of HIV-1 detection, although successfully established in lymphoid organs, have some limits to their sensitivity. Both immunohistochemistry and *in situ* hybridization are likely suitable to identify productive infection, *i.e.*, infection associated with viral replication and antigen expression. These techniques do not detect a true latent infection of cells, which might be detectable only by such sensitive techniques as PCR. The inability to detect HIV-1 RNA in more than one case with HIV-associated renal disease might further be explained by the limited tissue material that was available in some cases.

Renal parenchymal expression of the HIV cofactors CCR5 and CXCR4 is not upregulated in HIV-associated renal disease, whereas large numbers of infiltrating mononuclear leukocytes at sites of tubulointerstitial inflammation express CCR5 or CXCR4. Although productive HIV-1 infection of kidney parenchyma is undetectable, small numbers of HIV-1-infected mononuclear cells can be detected at sites of tubulointerstitial inflammation in kidneys with features of HIV-associated renal disease. Whether this finding is related to the pathogenesis of the renal injury and whether the presence of HIV in the kidney is capable of inducing cytopathic effects in susceptible individuals independent of direct renal infection, or whether it just represents nonspecific trapping of some HIV-infected leukocytes as "innocent bystanders" has not yet been ascertained (14). We believe that infected leukocytes circulating within the kidney, the altered cytokine milieu within the host occurring as a consequence of HIV infection and the development of AIDS, and certain host susceptibility factors such as racial background all contribute to the development of HIVAN. It has been difficult to directly test the importance of each of these variables in isolation, and hence the pathogenesis of HIV-associated renal disease remains far from being understood.

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