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Chemokine receptor (CCR5) expression in human kidneys and in the HIV infected macaque[1]

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Chemokine receptor (CCR5) expression in human kidneys and in the HIV infected macaque.

Background. The chemokine receptor, CCR5, has been identified as an essential co-receptor with CD4, which permits entry of human immunodeficiency virus (HIV) into mammalian cells. This receptor may also mediate leukocyte and parenchymal responses to injury by virtue of its binding to locally released chemokines such as RANTES, MIP-1α and MIP-1β during inflammation. The localization of CCR5 in human or primate kidney is unknown. In this study we sought to identify sites of CCR5 synthesis through localization of mRNA coding for this peptide.

Methods. CCR5 cDNA cloned into an expression vector was transcribed into a 1.1 Kb antisense riboprobe that was utilized for *in situ* hybridization (ISH) and Northern blotting studies.

Results. Northern analysis demonstrated positive hybridization for CCR5 mRNA in total RNA isolated from allograft nephrectomy tissue with features of severe transplant rejection as well as in kidney tissue with focal interstitial nephritis. No comparable hybridization signal was achieved with human kidney tissue uninvolved by disease. CCR5 mRNA was not identified in intrinsic renal cell types by ISH in normal human ($N = 6$), normal macaque kidney ($N = 5$), in kidneys from macaques with established infection by HIV-2 ($N = 9$), kidneys from macaques infected with HIV-1 ($N = 4$), nor in kidneys from SIV-infected macaques ($N = 5$). CCR5 was identified by ISH in human kidneys with features of interstitial nephritis ($N = 3$) and in rejected human allograft kidneys ($N = 14$). The expression of CCR5 was restricted to infiltrating mononuclear leukocytes at sites of chronic tubulointerstitial injury and at sites of vascular and interstitial rejection, respectively.

Conclusions. Understanding the localization of CCR5 as well as other chemokine receptors may help us understand how specificity in leukocyte trafficking is achieved in renal inflammatory processes such as allograft rejection and interstitial nephritis. They provide additional evidence that chemokines may be critical mediators of leukocyte trafficking in renal allograft rejection. These findings may account in part for the difficulty in demonstrating HIV infection of renal cells in human HIV infection, since these cells appear to lack constitutive expression of an essential co-receptor needed for viral entry.

[1] **See *Editorial* by Klotman, p. 2243.**

Key words: chemokine, graft rejection, HIV-associated nephropathy, transplantation, macrophages, receptors, CCR5.

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A recent review by Schlöndorff et al of chemokine activity in the kidney began with a fundamental question: "What determines the type of cellular infiltrate in tissue injury?" [1]. While many biochemical and physiologic signals are known to participate in this process, a central role has emerged which involves an expanding family of small molecules termed chemokines (chemotactic cytokines). Localized production and signaling by chemokines controls infiltration of tissues by specific classes of leukocytes through interactions with receptors borne by each leukocyte type [2, 3]. Most chemokines have been identified as belonging to one of two families, C-C and C-X-C, by virtue of whether or not an amino acid (X) is inserted between the first two conserved cysteine residues in individual molecules [2, 4]. While there is an apparent overlap in the binding properties and functions of many of the chemokines, there also appears to be some segregation of functions by families as manifest by the ability of these molecules to recruit specific classes of leukocytes (such as, monocytes vs. neutrophils) and perhaps mediate some additional biologic processes such as angiogenesis [2]. The existence of multiple receptors for chemokines offers a mechanism by which specificity in directing events in the inflammatory process by chemokines can be achieved.

The chemokine receptor CCR5 is one member of a family of structurally and functionally related seven-transmembrane-spanning, G-protein-coupled receptors. It is one of eight CC chemokine receptors that have been cloned and characterized for their ligand specificity [2, 4, 5]. CCR5 binds to three of the CC-chemokines, namely macrophage inflammatory protein-1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), and RANTES (regulated upon activation normal T cell expressed and secreted), but it does not bind monocyte chemoattractant protein-1 (MCP-1) [6, 7].

Considerable interest in renal expression of chemokines has proceeded from the recognition that they may mediate a variety of inflammatory injuries such as allograft rejection [1]. Recent observations in animal models and in human biopsy tissue have demonstrated an increased expression of CC-chemokines in solid organ transplants undergoing allograft rejection. Elevated expression of RANTES has been demonstrated in renal [8–10] and cardiac [11] allograft rejection, and the chemokines MIP-1α and MIP-1β were shown to be elevated in liver allograft rejection [12] and in the early phase of cardiac rejection [11]. It is likely that local expression of these chemokines is responsible for the interstitial and vascular mononuclear cell infiltrates of T cells and macrophages that characterize renal allograft rejection [13].

Interest in the chemokines and their receptors also comes from their recently identified participation in processes of cellular infectivity of human immunodeficiency virus (HIV) [14, 15]. New insights into the pathogenesis of HIV associated disease have been gained by the recognition that the chemokine receptor CCR5 serves as a major co-receptor together with CD4 for macrophage-tropic strains of HIV-1 in mammalian cells [16–20]. The specificity of T-lymphocyte-tropic strains of HIV-1 appears to result from their requirement for binding to the chemokine receptor CXCR4 as a condition of entry into mammalian cells [21, 22]. Expression of these molecules appears to be a key to understanding whether tissues are permissive for direct HIV infection. This has been substantiated by observations that genetic mutation of these molecules is a major impediment to HIV infection in individuals exposed to this virus [23–25].

In this study, we provide information on the cellular sites of synthesis of one chemokine receptor, CCR5, in mature adult human and primate kidneys, and in kidneys involved in allograft rejection and in immunodeficiency virus infection. In the case of renal allograft rejection, despite the growing numbers of reports on the role of chemokines in the rejection process, nothing is known about the expression of the corresponding receptors. We therefore studied whether a role for CCR5, the receptor for MIP-1α, MIP-1β and RANTES could be inferred in human renal allograft rejection.

Renal localization of CCR5 also has significance for understanding the pathogenesis of HIV nephropathy. It is still unknown whether the presence of intact virus or viral proteins within the renal parenchyma is required for the development of this nephropathy [26–28]. It has been proposed that HIV may directly infect renal parenchymal cells, although the data are conflicting. Biological heterogeneity in the strains of HIV-1 have been postulated to account for a particular "nephritogenic" strain [26]. HIV-1 strains isolated during the period of acute infection or from asymptomatic individuals are more often macrophage-tropic, whereas isolates from patients with fully developed AIDS are more often T-tropic or both macrophage and T-tropic. It has been hypothesized that macrophage-tropic strains characteristic of early disease states have a greater capacity to produce nephropathy [26]. We address the question as to whether or not CCR5, the major coreceptor for macrophage-tropic HIV-1 strains, is constitutively expressed by intrinsic renal cells in the belief that this would help our understanding of which cell types might be permissive for direct infection. Because non-human primates infected with simian immunodeficiency virus (SIV) [29] or, in some cases, with HIV-2, may be a model for human HIV associated nephropathy (HIVAN), we also sought to detect CCR5 mRNA expression in control and immunodeficiency virus infected primate tissues as well.

METHODS

Source of tissue

Normal human kidney tissue ($N = 9$) was obtained from kidneys surgically excised because of the presence of a localized neoplasm. Tissues utilized for this study were obtained from macroscopically normal portions of kidney located at some distance from the neoplastic process. Three of the nine kidneys showed the microscopic presence of focal interstitial nephritis with chronic tubulointerstitial injury and were subsequently regarded as a separate group. Normal kidneys from pig-tailed macaques, *Macaca nemestrina* ($N = 5$), sacrificed in the course of studies that did not involve initiation of disease processes, were obtained from the Regional Primate Research Center at the University of Washington.

In addition to these normal human and non-human primate kidneys, kidneys of macaques infected with different immunodeficiency virus strains were also utilized. Kidney tissue from pig-tailed macaques, *Macaca nemestrina*, infected with SIV_{Mne} ($N = 5$) [29], type D retrovirus ($N = 5$) [29], HIV-$2/_{287}$ ($N = 9$) [30], and HIV-1 ($N = 4$) [31, 32] were analyzed in this study. The detailed protocols for these studies have been published previously [29–32]. Animals were monitored for viremia and clinical disease. Animals developing an AIDS-like illness were euthanized when they were no longer able to be supported humanely by conventional veterinary care.

Human allograft nephrectomies ($N = 14$) excised for irreversible rejection were also utilized. All cases had features of severe cellular (interstitial) and vascular rejection. Many of these nephrectomy specimens also contained occasional, irregularly distributed lymphoid aggregates similar to those previously described in other solid organ allografts. The nephrectomy specimens in general were exposed to multiple courses of routine and intensified immunosuppression prior to excision, and represent a heterogenous sample from a clinical standpoint.

Portions of these tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin and sectioned. Additionally, unfixed tissue samples of normal human kidney, normal macaque kidney, spleen, and mesenteric

lymph node and human allograft nephrectomies were stored at −70°C prior to isolation of total RNA.

Cell lines

To confirm the specificity of the CCR5 riboprobe, chemokine receptor transfected cell lines were used as controls. HOS cells expressing CCR1, CCR2B, CCR3, CCR4, CCR5, or CXCR4 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau [16, 33]. The cells were grown in DMEM medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum and 0.5 μg/ml puromycin (Fluka, Ronkonkoma, NY, USA). After reaching confluency, the cells were trypsinized and centrifuged. The resulting cell pellets were either fixed in 10% phosphate-buffered formalin, paraffin embedded and analyzed by *in situ* hybridization or used for total RNA isolation.

Molecular probes

The CCR5 expression vector was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau) and has been characterized previously [16, 34]. The 1.1 Kb insert was derived from cDNA encoding CCR5, and cloned into pcDNAI/amp (Invitrogen, Carlsbad, CA, USA).

The plasmid was cut with appropriate restriction enzymes and transcribed into antisense and sense riboprobes, using reagents from Promega (Madison, WI, USA), except [^{35}S]-uridine triphosphate (UTP), which was obtained from New England Nuclear (Boston, MA, USA). The transcription reaction mixture contained 1 μg of CCR5 cDNA (sense or antisense orientation), 250 μCi of [^{35}S]-UTP, 500 μmol/liter each of adenosine 5′-trisphosphate (ATP), cytidine 5′-triphosphate (CTP) and guanosine 5′-triphosphate (GTP), 40 U of RNAsin, 10 mmol/liter dithiothreitol, 40 mmol/liter Tris and 10 U of either SP6 or T7 polymerase. After incubating 75 minutes at 37°C, the template DNA was digested by adding 1 U of RQ1 DNAse (Promega) and incubated at 37°C for an additional 15 minutes. Free nucleotides were separated with a Sephadex G-50 column. The collected fraction containing labeled probe was then ethanol precipitated. The probe was resuspended in nuclease free water containing 10 mmol dithiothriotol, counted and used within 48 hours. The CCR5 antisense riboprobe generated for the Northern analysis was labeled with [^{32}P]-UTP (New England Nuclear).

A 1.7 kb sequence of DNA coding for HIV-2 gp120 (*env*) (nucleotides 6480-8335 of the HIV-2/$_{287}$ sequence), cloned into pCR II (Invitrogen), was kindly provided by Bristol-Myers Squibb (Seattle, WA, USA). Antisense and sense riboprobes were generated for *in situ* hybridization following the above mentioned protocol. Specificity of the HIV-2 gp120 riboprobe was established by *in situ* hybridization. PBMC from HIV-2/$_{287}$ infected macaques and from uninfected macaques were fixed in formalin, paraffin embedded and sections of the fixed PBMC pellets were analyzed by *in situ* hybridization. Positive hybridization with the HIV-2/$_{287}$ gp120 antisense probe could be detected only for the cells from HIV-2 infected animals. Furthermore, no hybridization signal was demonstrable on lymph nodes obtained from uninfected, normal macaques ($N = 5$).

The pTRI-RNA-28S antisense control template containing a 115 bp cDNA fragment of the human 28S rRNA gene was purchased from Ambion (Austin, TX, USA). Run-off transcripts to total RNA detect the 4,718 nucleotide 28S rRNA species in a Northern blot. The *in vitro* transcription reaction was performed with Digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, IN, USA) and T7 polymerase.

In situ hybridization

Tissue and cells were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned, and deparaffinized following standard protocols which we have previously utilized [35, 36]. The sections were washed with 0.5 × standard saline citrate (SSC) (1 × SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0) and digested with proteinase K (5 μg/ml; Sigma) in Tris buffer for 30 minutes at 37°C. Several 0.5 × SSC washes were followed by prehybridization for two hours in 100 μl of prehybridization buffer (0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, 10 mM DTT). 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 washed with 0.5 × SSC, treated with RNase A (20 μg/ml, 30 min at 37°C), washed in 2 × SSC (2 × 2 min), followed by three high-stringency washes in 0.1 × SSC/0.5% Tween 20 (Sigma) for 40 minutes 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, USA) and exposed in the dark at 4°C for four weeks (CCR5), or two weeks (HIV-2), respectively. After developing, the sections were counterstained with hematoxylin and eosin, dehydrated and coverslipped.

RNA isolation

Total RNA was extracted from frozen tissue and chemokine receptor transfected cell lines, respectively, using the Total RNA Isolation Kit (Ambion).

Briefly, unfixed frozen tissue was disrupted by grinding it to a powder and then it was denatured using a tissue homogenizer (Tissumizer SDT-1810; Teckmar, Cincinnati, OH, USA). Following phenol/chloroform extraction, the RNA was recovered by isopropanol precipitation. Remaining salt was removed by ethanol wash and residual carbohydrate by lithium chloride precipitation. Finally, the pellets were resuspended in RNase-free water. The RNA concentration was measured by reading the absorbance in a spectrophotometer at the wavelength of 260 nm. The

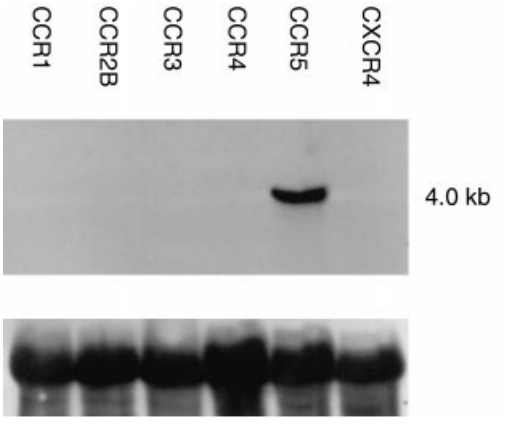

Fig. 1. CCR5 Northern blot of transfected HOS cell lines demonstrating specificity of the probe for CCR5 mRNA. Ten micrograms of total RNA, isolated from different chemokine receptor transfected cell lines, were electrophoresed and blotted as described in the methods section. Hybridization with the CCR5 riboprobe (*top*) detects a single band at the expected size of 4.0 kilobase only in RNA isolated from CCR5 transfected cells. Hybridization for 28S rRNA (*bottom*) demonstrates comparable amounts of total RNA in each lane.

intactness and overall quality of the total RNA preparation was assessed by electrophoresis on a native agarose gel demonstrating sharp and intense 28S and 18S ribosomal bands.

Northern analysis

Samples (10 μg RNA) were electrophoresed through a 1% agarose-formaldehyde gel and transferred to a positively charged nylon membrane (BrightStar-Plus; Ambion). Blots were hybridized overnight with the [^{32}P]-UTP labeled CCR5 riboprobe as described previously and exposed in the dark at −70°C for 10 days. To confirm the intactness and total amount of the blotted RNA the membranes were subsequently stripped and hybridized with the Digoxigenin-UTP labeled 28S riboprobe, using the Northern Max Northern blotting kit (Ambion).

RESULTS

Molecular probe

Specificity of the CCR5 antisense riboprobe was established by Northern analysis and *in situ* hybridization. Total RNA isolated from HOS cell lines transfected with DNA coding for CCR5, CCR1, CCR2B, CCR3, CCR4, and CXCR4 respectively was generated for Northern analysis. Positive hybridization with the CCR5 riboprobe could only be detected for the CCR5 transfected cell line showing a single band at the expected 4.0 kilobase size of CCR5 mRNA (Fig. 1). No nonspecific crosshybridization with RNA isolated from the several control cell lines could be detected.

Paraffin embedded cell pellets of the CCR5 transfected HOS cell line and of similar cell lines transfected with CCR1, CCR2B, CCR3, CCR4 and CXCR4 respectively were utilized for *in situ* hybridization. Positive hybridization with the CCR5 antisense probe was demonstrable on the CCR5 cell line (Fig. 2A), but not on any of the other cell lines transfected with different chemokine receptors (data not shown). Additional negative controls included the absence hybridization when identical procedures were performed with substitution of a sense probe for hybridization (Fig. 2B).

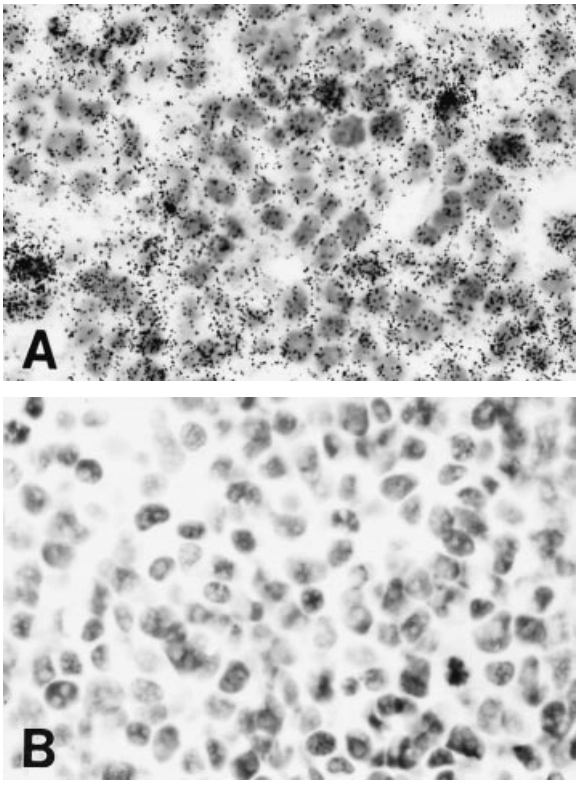

Fig. 2. *In situ* hybridization of transfected HOS cell lines demonstrating specificity of the probe for CCR5 mRNA. (*A*) Positive hybridization with the CCR5 antisense riboprobe is demonstrable on a formalin fixed, paraffin embedded pellet of CCR5 transfected HOS cells. (*B*) No hybridization of the control CCR5 sense riboprobe is detectable on the same CCR5 transfected cell pellet.

Normal kidneys

CCR5 mRNA was undetectable by Northern analysis in total RNA isolated from normal human (Fig. 3) and macaque (data not shown) kidneys, respectively. This result was confirmed by *in situ* hybridization for CCR5 mRNA in formalin fixed paraffin embedded normal kidney tissue. *In situ* hybridization showed the presence of a very small number of CCR5 mRNA expressing cells in normal human kidneys (Fig. 4A). Those cells, which appeared to be leukocytes, were localized in the interstitium, at times demonstrable within the lumina of peritubular capillaries. No cells which could be clearly identified as intrinsic renal cells within the glomerular, tubular, interstitial, or vascular compartments showed positive hybridization for CCR5. CCR5 mRNA expression was completely absent in normal

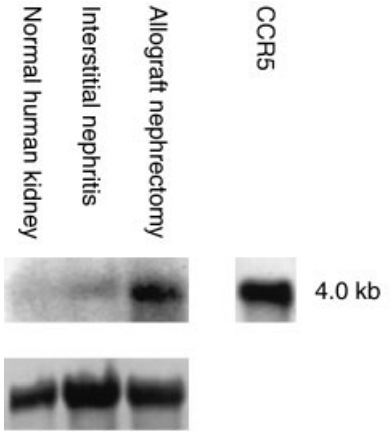

Fig. 3. CCR5 Northern blot of different kidney tissues. Ten micrograms of total RNA, isolated from frozen kidney tissue, were electrophoresed and blotted as described in the Methods section. CCR5 mRNA is detected in total RNA isolated from one human allograft nephrectomy, and a weak band is present in total RNA isolated from one human kidney with features of focal interstitial nephritis. Normal human kidney RNA shows no detectable hybridization signal for CCR5 mRNA. The positive control was performed on the same membrane and shows hybridization for RNA isolated from CCR5 transfected cells. Control hybridization for 28S rRNA (*bottom*) was performed on the same membrane and confirmed comparable amounts of loaded total RNA.

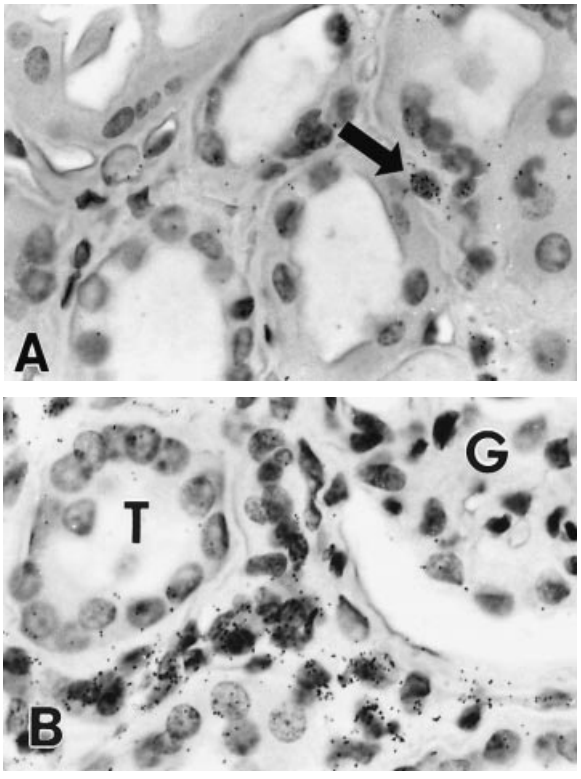

Fig. 4. CCR5 expression in native human kidney. (*A*) Very few CCR5 mRNA expressing cells are detectable in normal human kidneys. *In situ* hybridization for CCR5 mRNA shows occasional positive mononuclear cells in the lumina of peritubular capillaries and in the interstitium (arrow). (*B*) Human kidneys with features of focal interstitial inflammation demonstrate CCR5 mRNA expression at sites of leukocytic infiltration. CCR5 mRNA is not detectable in the glomerular (G) or tubular (T) compartment. Original magnification ×1000.

macaque kidneys by *in situ* hybridization (data not shown). Three of the macroscopically normal kidneys utilized demonstrated the mild nephrosclerosis characteristic of aging kidneys as well as the focal interstitial inflammation typically associated with renal fibrosis, and were considered as a separate group. Northern analysis detected a weak hybridization signal for CCR5 mRNA in the RNA isolated from one human kidney with such interstitial inflammation (Fig. 3). By *in situ* hybridization CCR5 mRNA expression was occasionally demonstrable at sites of leukocytic infiltration associated with chronic tubulointerstitial injury (Fig. 4B). The hybridization signal was confined to infiltrating mononuclear cells and could not be detected on tubular or glomerular cells or on vascular endothelium.

Animal models of AIDS

The CCR5 antisense probe utilized in this study was originally generated against human CCR5. We were able to show that this riboprobe also detects non-human primate CCR5. *In situ* hybridization demonstrated the presence of CCR5 expressing cells in lymph node (Fig. 5A) and spleen (data not shown) tissue isolated from HIV-2 infected macaques. The expression of the chemokine receptor CCR5 correlated with HIV-2 infection of macaque lymph nodes, as shown by positive *in situ* hybridization for HIV-2 gp120 RNA in sequential sections of the same lymph node (Fig. 6).

In kidneys of HIV-2 ($N = 9$) or HIV-1 ($N = 4$) infected macaques, hybridization for CCR5 mRNA was completely absent (Fig. 5B). None of these animals showed specific renal pathologic lesions similar to those described in HIV-infected humans. Kidneys obtained from SIV_{Mne} and type D retrovirus infected macaques also revealed the absence of CCR5 mRNA hybridization signal by *in situ* hybridization (data not shown).

Human renal allograft rejection

Northern analysis detected CCR5 mRNA in total RNA isolated from the one allograft nephrectomy studied in this manner (Fig. 3). The histopathological evaluation of this kidney demonstrated features of severe cell-mediated transplant rejection. All fourteen allograft nephrectomies included in this study demonstrated severe cell-mediated transplant rejection with variable degrees of vascular rejection. This was characterized by diffuse, at times massive, interstitial mononuclear cell infiltrates and arterial intimal proliferative changes with inflammatory cell infiltration as previously described and illustrated [35]. CCR5 mRNA expressing cells were detectable in all observed allograft nephrectomies, but were variable in number in the different cases (Fig. 7 A, C). Nine nephrectomies exhibited a large number of CCR5 mRNA expressing cells, and in five kidneys the number of such cells was relatively few.

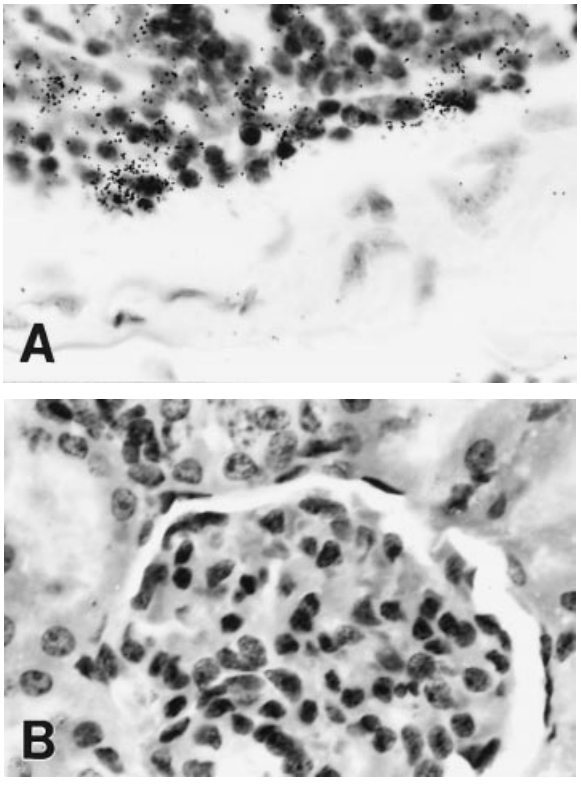

Fig. 5. CCR5 expression in the HIV-2 infected macaque. (*A*) CCR5 antisense riboprobe also detects non-human primate CCR5 mRNA. CCR5 expressing cells are detected in mesenteric lymph nodes isolated from pig-tailed macaques infected with HIV-2/$_{287}$. (*B*) CCR5 mRNA is not detected in renal parenchyma of macaques infected with different immunodeficiency viruses. *In situ* hybridization for CCR5 mRNA showed no positive cell in a kidney of a HIV-2/$_{287}$ infected pig-tailed macaque. Original magnification ×1000.

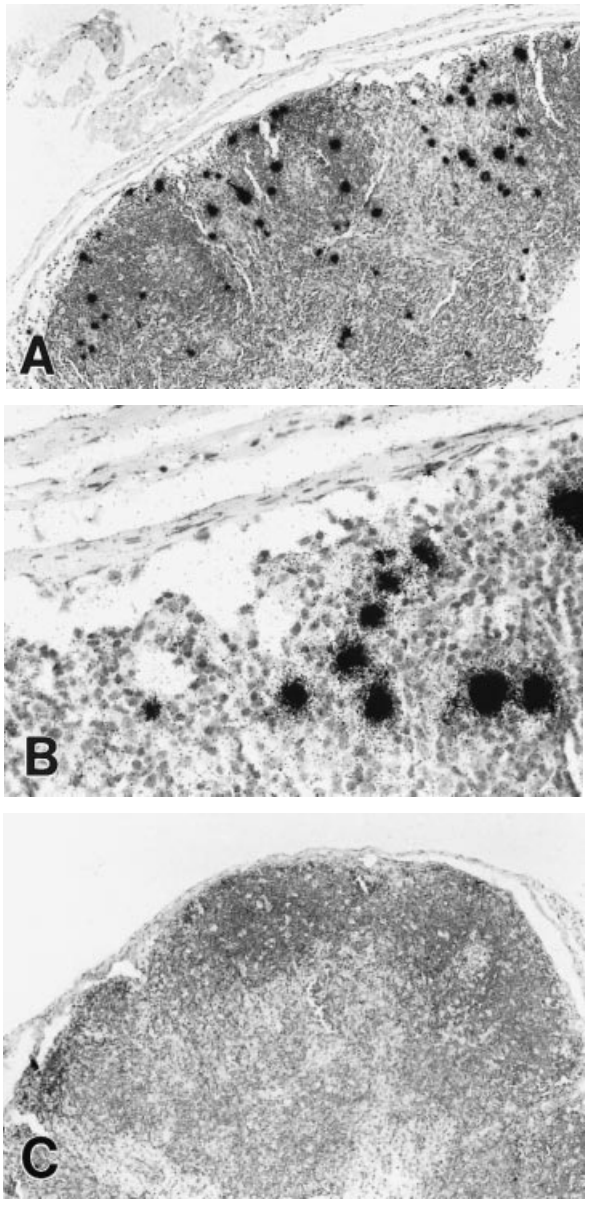

Fig. 6. HIV-2 RNA in infected macaque lymph nodes is demonstrable in lymph nodes expressing CCR5 mRNA. (*A*) *In situ* hybridization for HIV-2/$_{287}$ gp120 RNA detects HIV-2 infected cells in mesenteric lymph nodes of HIV-2/$_{287}$ infected macaques, seen at higher magnification in (*B*). This is a sequential section of the same lymph node illustrated in Figure 5A. (*C*) Absent hybridization on the same HIV-2 infected mesenteric lymph node is demonstrable with substitution of a sense probe for hybridization. Original magnification A and C ×100, B ×400.

The expression of CCR5 was restricted to infiltrating mononuclear cells. A few CCR5 positive cells were detected in the capillary lumina of some glomeruli (Fig. 7B), but CCR5 mRNA expression by intrinsic glomerular cells could not be detected. Hybridization signal for CCR5 mRNA was absent in tubular cells and in endothelial cells. In nephrectomies with additional features of vascular rejection, some neointimal cells showed CCR5 mRNA expression (Fig. 7E). Assessment of whether these cells expressing CCR5 mRNA were infiltrating macrophages/lymphocytes or neointimal smooth muscle cells was not possible in these cases, because of technical limitations we have encountered with the sensitivity of the radioactive hybridization technique using this particular CCR5 probe. The sensitivity of this procedure becomes markedly compromised when additional immunohistochemical double labeling procedures are added.

DISCUSSION

We were unable to demonstrate constitutive expression of the chemokine receptor CCR5 in intrinsic renal cells using a riboprobe with specificity for CCR5 mRNA. Neither normal human or macaque kidneys nor kidneys obtained from macaques infected with several different immunodeficiency viruses demonstrated CCR5 mRNA expression. However, we were able to detect for the first time the presence of CCR5 expressing cells in the kidney, in the setting of various inflammatory disease processes. We detected CCR5 at sites of leukocytic infiltration in cases of focal interstitial nephritis and in renal allograft nephrectomies with features of severe transplant rejection.

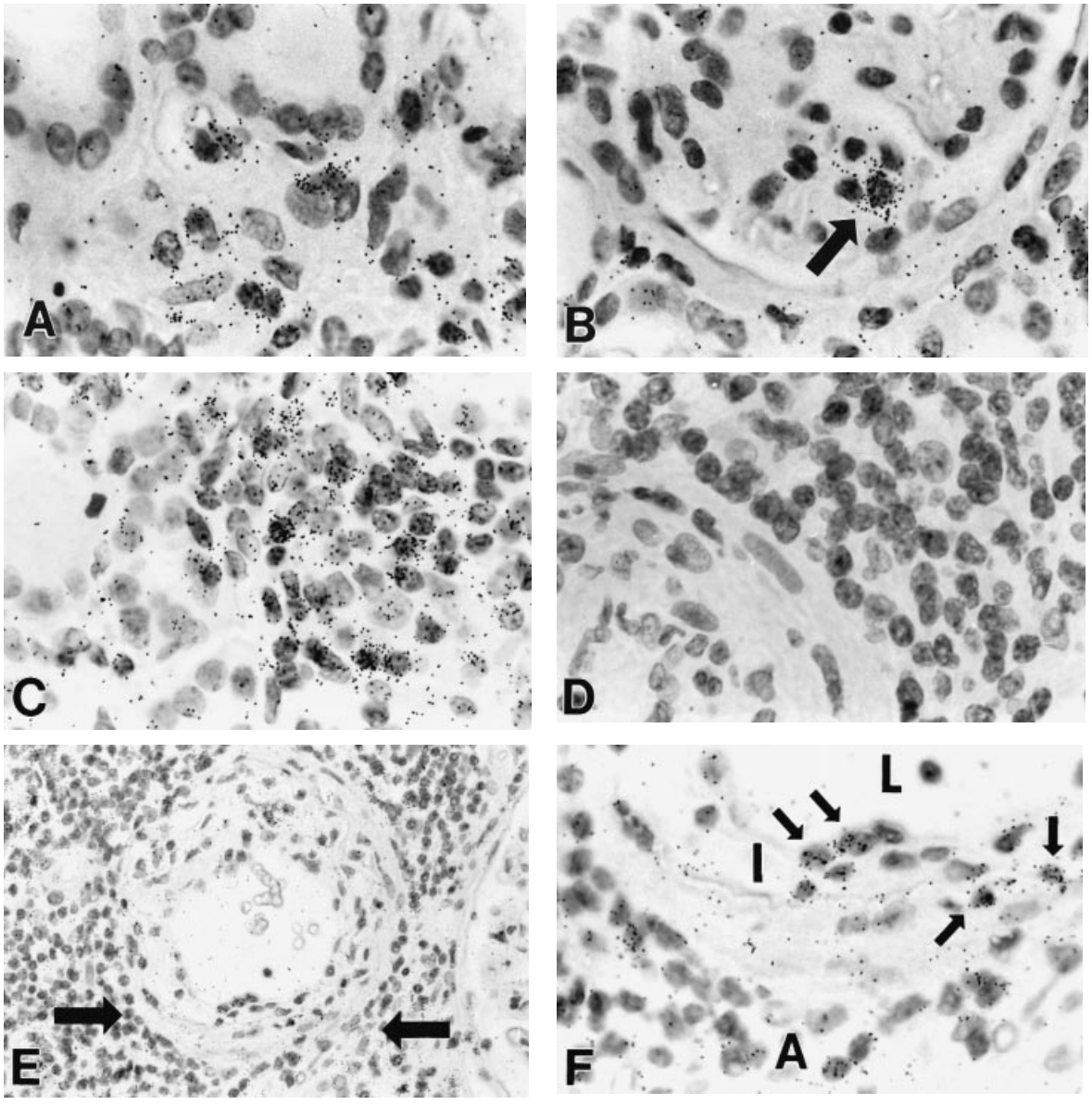

Fig. 7. CCR5 expression in human renal allograft rejection. (*A*) CCR5 mRNA expression is detectable in all observed allograft nephrectomies and is restricted to infiltrating mononuclear cells in the interstitium and in rejecting arteries. Tubular cells do not demonstrate a positive CCR5 hybridization signal. (*B*) A small number of CCR5 positive cells can be detected in the capillary lumina of some glomeruli (arrow), but intrinsic glomerular cells do not show a hybridization signal for CCR5 mRNA. (*C*) A large number of CCR5 mRNA expressing cells is detectable at sites of interstitial mononuclear cell infiltrates. (*D*) No hybridization signal of the CCR5 sense control riboprobe is detectable on a cellular infiltrate of the same human allograft nephrectomy. (*E*) Allograft nephrectomies with additional features of vascular rejection occasionally demonstrate some CCR5 mRNA expressing neointimal cells. The area between arrows is illustrated in higher power in panel F. (*F*) Examples of CCR5 mRNA expressing neointimal cells in a rejecting artery are indicated by arrows. CCR5 mRNA expression is also demonstrable in several mononuclear cells within the perivascular/adventitial infiltrate illustrated in E. Abbreviations are: L, lumen; I, neointima; A, adventitia. Original magnification A-D and F $\times$1000, E $\times$400.

Chemokines and the corresponding receptors play a central role in regulation of inflammatory processes [2, 3, 37, 38]. To further define a possible role of the chemokine receptor CCR5 in renal inflammatory disease we studied the expression of CCR5 mRNA in renal allograft rejection. Cell-mediated transplant rejection is represented by an interstitial mononuclear infiltrate of T cells, macrophages, and occasionally eosinophils [13]. Several studies reported an elevated expression of the CCR5 ligands RANTES, MIP-1α and MIP-1β in solid organ transplants with features of cell-mediated rejection [8–12]. It has been hypothesized that infiltrating mononuclear cells and intrinsic parenchymal cells within inflammatory foci locally express specific chemokines. These chemokines may then be responsible for inducing adhesive interactions between rolling leukocytes and the endothelium, subsequently attracting diapedesing leukocytes to specific tissue sites of inflammation by a chemokine concentration gradient [3, 38,

39]. Specificity is achieved by virtue of the ability of the chemokines produced at a given site of injury to bind to specific receptors, such as CCR5, borne on the surface of different classes of leukocytes.

This study demonstrates the presence of CCR5 expressing cells in renal allograft rejection using two complementary approaches. Northern analysis demonstrated an intense hybridization signal for CCR5 mRNA in a kidney with severe transplant rejection. This initial result was confirmed by *in situ* hybridization studies. All observed cases with the diagnosis of severe transplant rejection exhibited an increased expression of CCR5 mRNA. The presence of this chemokine receptor was restricted to infiltrating mononuclear cells and was not seen on intrinsic renal cells. We did observe variability in the extent of synthesis of CCR5 mRNA between kidneys with similar degrees of inflammatory cell infiltration. While we have no data that would clearly account for this observation, some possibilities include irregular synthesis of CCR5 (that is, discontinuous vs. steady-state production) and the possibility that other chemokine receptors may be more important in mediating sites of inflammatory cell aggregation in some allograft kidneys, as discussed below. Our observations in allograft kidneys are supported by the related observation that CCR5 mRNA expression is also detectable in infiltrating inflammatory cells in kidneys with features of focal interstitial nephritis.

Immunohistochemistry for CCR5 protein performed by Rottman et al in one kidney with features of interstitial nephritis showed positive staining for rare interstitial mononuclear cells and also arteriolar endothelial and vascular smooth muscle cells [40]. Using the alternate technical approach of *in situ* hybridization, we were unable to show CCR5 mRNA expression on either endothelial or vascular smooth muscle cells in this study. The monoclonal antibody against CCR5 protein used by Rottman et al was not available for our study.

One other recent study focused on chemokine receptor expression in kidney tissue. Schadde et al detected CCR 1, 2, 3, and 5 in RNA isolated from normal mouse kidneys by quantitative RT-PCR [41]. After experimental induction of an anti-glomerular basement membrane nephritis in these mice, an increase of mRNA expression for CCR1, 2, 3, and 5 was detected. Due to the lack of a morphological correlate in the design of this study one cannot determine whether some or all of the increased chemokine receptor expression reflects an increased number of chemokine receptor expressing infiltrating macrophages and/or lymphocytes involved in the pathogenesis of this glomerulonephritis, or is the result of *de novo* production by renal parenchymal cells.

Further studies focusing on the role of other chemokine receptors are necessary to understand the complicated network of leukocyte attraction in the pathogenesis of renal transplant rejection. Although we were able to detect a strong expression of CCR5 mRNA in most kidneys with severe allograft rejection, it is important to point out that a large percentage of infiltrating leukocytes did not express CCR5. It is likely that different chemokine receptors are expressed on CCR5 negative infiltrating cells. An upregulated expression of another CC-chemokine, monocyte chemoattractant protein-1 (MCP-1) has also been demonstrated in renal allograft rejection [42, 43]. MCP-1 is a ligand for CCR2 and CCR4 [2, 4]. To our knowledge, no study has analyzed the expression of these chemokine receptors in renal allograft rejection. With better definition of chemokine mediated leukocyte trafficking events, we believe new therapeutic approaches in renal allograft rejection using anti-chemokine agents may be envisioned.

The second component of this study bears on the role of CCR5 in the pathogenesis of HIV associated injury. During the past two years, chemokine receptors have been identified as essential co-receptors for human immunodeficiency virus infection [14, 15]. Macrophage-tropic strains of HIV-1 involved in the initial infection phase utilize CCR5 as a major co-factor. The binding of HIV-1 envelope protein gp120 to the cell surface receptors CD4 and chemokine receptor CCR5 subsequently permits entry of HIV-1 into the cell [14, 15]. CCR3 and CCR2B have similar functions, but their role as co-receptors is less prominent, suggesting that viral envelope proteins have lower affinity for these receptors. T lymphocyte-tropic strains of HIV-1 isolated from patients with clinical AIDS more frequently utilize CXCR4 as infection co-receptor. The involvement of chemokine receptors in the infection process has been further supported by *in vitro* inhibition studies. Infection of macrophage-tropic HIV-1 strains can be suppressed by the CCR5 ligands MIP-1α, MIP-1β and RANTES [17, 19, 44], and the CXCR4 ligand SDF-1 was found to be a potent inhibitor of infection by T-tropic HIV-1 strains [45, 46].

Although all of the initial reports on chemokine receptors as HIV co-receptors represent *in vitro* findings, further *in vivo* observations support the role of this receptor family in the pathogenesis of HIV infection. A mutant allele of the CCR5 gene bearing a 32-nucleotide deletion (CCR5 delta 32) has recently been characterized [23, 24] and individuals homozygous for this allele have been linked to protection from HIV-1 infection [47, 48]. The consequence of the heterozygous state is not clear, but it may delay the progression to AIDS in infected individuals [47–49].

HIV-associated nephropathy is a unique disease that can be seen in up to 10% of some HIV-infected populations. Characteristic histologic features of HIV-associated nephropathy (HIVAN) include a collapsing form of focal and segmental glomerulosclerosis and microcystic dilation of renal tubules [26, 28]. Whether the glomerulopathy that occurs in HIVAN may be a direct consequence of infection with HIV or a result of exposure to select viral peptides or whether it may be due to other factors independently present in certain patient populations is unknown. While

there is evidence from a single publication that HIV can directly infect renal parenchyma as demostrated by *in situ* hybridization [50], we (unpublished observations) and others [50, 51] have not been able to replicate this result. In previous studies we have not identified specific antigens or messenger RNA of HIV (unpublished observations) or SIV [29] in renal parenchymal cells in primate models of immunodeficiency virus infection. Investigations into the infectivity of human glomerular cells *in vitro* have yielded conflicting results. We have been unable to demonstrate productive infection of human mesangial cells by either T-tropic or macrophage-tropic strains of HIV-1 and HIV-2 [53]. One other group has reported HIV infectivity *in vitro* of human glomerular endothelial cells and a small proportion of mesangial cells but not epithelial cells [54]. However, the experimental design of that study suggested the possibility of transfection rather than infection of renal cells.

This present study sought to detect the expression of the HIV co-receptor CCR5 in normal kidneys and in kidneys obtained from primates infected with different immunodeficiency viruses. *In situ* hybridization failed to detect expression of CCR5 mRNA on renal parenchymal cells in normal human and non-human primate kidneys. A small number of CCR5 expressing cells could be seen in normal kidney tissue within the lumina of peritubular capillaries and showed morphological features of lymphocytes or monocytes. There was no evidence for the presence of CCR5 mRNA on mesangial cells, vascular endothelial cells, vascular smooth muscle cells, glomerular epithelial cells or interstitial fibroblasts. No CCR5 mRNA expression in primate kidneys was detectable when inflammatory cells were absent.

The absent expression and/or detection of CCR5 mRNA in uninfected normal renal tissue does not exclude a role for CCR5 in HIV-associated renal disease. An up-regulation of chemokine receptor expression might be detectable in disease states. We therefore additionally investigated whether CCR5 could be detected in animal models of AIDS. The riboprobe generated against human CCR5 crossreacts with non-human primate CCR5. Pig-tailed macaques infected with HIV-1 [31, 32] and a strain of HIV-2 ($HIV\text{-}2/_{287}$) [30] that induces an AIDS-like disease failed to develop specific renal pathologic lesions similar to those of HIVAN (F. Eitner, unpublished observations). Furthermore, we were not able to detect CCR5 mRNA expression in the kidney of HIV-1 or HIV-2 infected macaques, nor in the kidney of SIV_{Mne} infected macaques [29]. Perhaps as a consequence of absent parenchymal expression of CCR5, we found that kidney tissue of these primates infected with HIV-2 exhibited no messenger RNA encoding for HIV-2 viral proteins as demonstrated by *in situ* hybridization (manuscript in preparation), indicating the absence of parenchymal infection. We believe this failure to detect specific viral RNA and CCR5 mRNA in renal parenchyma of macaques infected with different immunodeficiency viruses provides additional insight into the difficulty encountered in attempts to demonstrate direct infection of intrinsic renal cells by immunodeficiency viruses.

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