Expression of Chemokines and Chemokine Receptors During Human Renal Transplant Rejection

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● Infiltration of renal allografts by leukocytes is a hallmark of acute transplant rejection. Chemokines attract leukocytes bearing specific chemokine receptors, and the specific leukocyte chemokine receptor phenotype is associated with types of immune responses, ie, T helper subtype 1 (Th1; CXC chemokine receptor 3 [CXCR3], CC chemokine receptor 5 [CCR5]) versus Th2 (CCR3, CCR4, CCR8). We studied the expression of the chemokine monocyte chemoattractant protein-1 and the chemokine receptors CCR2B and CXCR4 messenger RNA (mRNA) by in situ hybridization, as well as the chemokine receptors Duffy antigen receptor for chemokines (DARC) and CCR5 protein by immunohistochemistry in renal biopsy specimens with acute cellular rejection (n = 12) and acute vascular rejection (n = 8), transplant nephrectomy specimens (n = 6), and normal areas of tumor nephrectomy specimens (n = 5). CC chemokines and CC chemokine receptor mRNA expression were evaluated by ribonuclease protection assay in specimens from four transplant nephrectomies and one tumor nephrectomy. Upregulation of mRNAs for the chemokines, interferon-inducible protein-10 (IP-10); regulated on activation normal T-cell expressed and secreted; macrophage inflammatory protein-1α (MIP-1α); MIP-1β; and lymphotactin, as well as the chemokine receptors, CCR2 and CCR5, were documented during allograft rejection. CCR1 mRNA was detectable in both allografts and controls, but CCR3 and CCR8 were absent. The number of CXCR4, CCR5, and CCR2B mRNAs expressing leukocytes and DARC-positive vessels increased during rejection episodes. CXCR4 mRNA was the most widely expressed. Leukocytes in diffuse interstitial infiltrates were mainly CCR5 positive, but in areas in which leukocytes formed nodular aggregates of infiltrating cells, the number of CCR5-positive cells was low. Instead, leukocytes in these nodular aggregates mainly expressed CXCR4. DARC was expressed on peritubular capillaries, where it was upregulated in areas of interstitial infiltration. Induction of chemokines during renal allograft rejection is accompanied by infiltration of leukocytes bearing the respective chemokine receptors. The upregulation of the CXCR3 ligand IP-10, as well as CCR5 and its ligands, in the absence of CCR3 and CCR8 is indicative that renal allograft rejection is primarily the result of a Th1-type immune response.
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INDEX WORDS: Chemokines; chemokine receptors; renal allograft rejection; Duffy antigen receptor for chemokines (DARC); immune response; T helper subtype 1 (Th1).

R EJECTION REMAINS a major obstacle to long-term survival of renal allografts.[1,2] Acute rejection is manifested by leukocyte infiltration of different compartments of the transplanted kidney.[3,4] The main types of infiltrating

cells are subsets of T cells and macrophages at different stages of activation.[5-7] Distinct immune responses against different challenges are termed T helper subtype 1 (Th1)-like or Th2-like according to the classes of Th cells involved.[8,9] These subtypes of Th cells are characterized by the expression patterns of cytokines and chemokine receptors.[8-11] A Th1 response resulting in activation of cytotoxic T cells and delayed-type hypersensitivity is believed to be important during transplant rejection.[12,13]

The role of chemokines, a family of small chemotactic cytokines, in various inflammatory responses has become apparent during the last decade.[14-17] Several studies described the expression and potential role of chemokines and chemokine receptors during renal transplant rejection (reviewed in[14]). Data about chemokine-receptor expression during human transplant rejection are still scarce.[18-21] In this study, we describe the morphological distribution of four chemokine receptors potentially involved in allograft infil-

tration by T cells and macrophages. CXCR4-positive cells, mainly T cells, have been shown to be involved in human renal allograft rejection.[19] CCR5 is a receptor for the chemokines regulated on activation normal T-cell expressed and secreted (RANTES); macrophage inflammatory protein-1α (MIP-1α); MIP-1β; and monocyte chemoattractant protein-2 (MCP-2). Our previous studies imply that CCR5-positive cells might have an important role during interstitial infiltration.[18,21] The distribution of cells expressing CCR2B (a receptor for MCP-1) during human renal transplant rejection is currently unknown. CXCR4, CCR5, and CCR2 are expressed on different subsets of T cells and monocytes/macrophages.

In this study, we show the upregulation of several CC chemokines and their corresponding receptors, consistent with a Th1-type reaction, within the allograft kidney. In addition, we describe localized patterns of leukocyte accumulation that correspond to distinct patterns of chemokine-receptor expression and are consistent with subpopulations of leukocytes being attracted by different chemokines. Finally, we show the expression of the promiscuous chemokine receptor, Duffy antigen receptor for chemokines (DARC), which shares ligands with CCR5 and CCR2 and is expressed on interstitial capillaries at sites where the extravasation of leukocytes might take place in part.

METHODS

Renal Specimens

Thirty-one renal specimens were examined. Included in the study were specimens from transplant nephrectomies (n = 6) with acute cellular rejection only (n = 2) and acute cellular and vascular rejection (n = 4). Renal transplant biopsy specimens included those with acute cellular rejection (n = 12) and acute vascular rejection (n = 8). Normal areas of tumor nephrectomy specimens served as controls (n = 5). Renal biopsy specimens were from cases studied between 1995 and 1997 in the Department of Pathology, University of Washington (Seattle, WA). Biopsy specimens from patients with acute cellular or vascular rejection were included when sufficient material for multiple immunohistochemistry and in situ hybridization studies was available after routine diagnostic workup was completed. The nephrectomy specimens were collected during 1998 and 1999. Sufficient frozen material for RNA isolation was available in five nephrectomy cases, including a tumor nephrectomy (n = 1) and renal allograft nephrectomies with acute cellular rejection (n = 2) and acute vascular rejection (n = 2). Approval of the University of Washington Internal Review Board for Human Subjects prescribed that no patient identifiers may be linked to studies involving nephrectomy or biopsy tissue; therefore, clinical data were not available for morphological correlations.

RNA Isolation

Total RNA was isolated applying the ToTALLY RNA total RNA isolation Kit (Ambion, Austin, TX) according to the instructions of the manufacturer. Frozen tissue was disrupted on dry ice and homogenized by a tissue homogenizer (Tissumizer SDT-1810; Teckmar, Cincinatti, OH) in 10 mL of denaturation solution per gram of tissue. After vigorous mixing with one volume of Phenol/CHCl$_3$ Solution #1 (Ambion), the solution was incubated for 15 minutes on ice. Phases were separated by centrifugation. One tenth of the volume of 3 mol/L of sodium acetate and one volume of Phenol/ChCl$_3$ Solution #2 (Ambion) were added to the aqueous phase. After another incubation and centrifugation, RNA was recovered from the aqueous phase by isopropanol precipitation. Pellets were washed with 70% ethanol and resuspended in ribonuclease (RNase)-free water. RNA concentration was calculated according to absorbance in a spectrophotometer at a wavelength of 260 nm.

RNase Protection Assay

The RiboQuant (Pharmingen, San Diego, CA) system was used for the comparison of chemokine and chemokine receptor messenger RNA (mRNA) expression by RNase protection assay (RPA). The templates for human CC chemokine receptors included CCR1-5 and CCR8 (hCR-5; Pharmingen), and the chemokine template included lymphotactin, RANTES, interferon-inducible protein-10 (IP-10), MIP-1α, MIP-1β, MCP-1, interleukin-8, and I309 (a chemokine that binds to CCR8). The probe synthesis was performed in a total volume of 20 μL containing 100 μCi of α-phosphorus 32–labeled uridine triphosphate, 40 U of RNasin, 10 mmol/L of dithiothreitol (DTT), 1 $\times$ transcription buffer, guanidine-adenine-cytosine-uracil (GACU) pool, 20 U of T7 polymerase, and the RPA template set (Pharmingen). After 1 hour at 37°C, the reaction was stopped by digestion of the template with 2 U of RNase-free deoxyribonuclease for 15 minutes. Phenol extraction and ethanol precipitation were performed, and the pellet was washed with 90% ethanol. The air-dried pellet was dissolved in 50 μL of hybridization buffer. RNA probes were dried in a vacuum evaporator centrifuge for 1 hour (15 μg of RNA for chemokine receptors, 5 μg of RNA for chemokines). Two microliters of probe diluted in hybridization buffer to 2.8 cpm/μL (chemokine receptors) or 3.1 counts per minute (cpm)/μL (chemokines) were added to each RNA vial and incubated overnight at 56°C. The probes were treated with RNase A+T1 mix (Pharmingen) at 30°C for 45 minutes, followed by a proteinase K digestion for 15 minutes at 37°C. After another phenol extraction and ethanol precipitation, the probes were air dried, redissolved, and separated on a 5% acrylamide gel. The gel was dried on a gel drier and exposed to film for 2 to 7 days.

Molecular Probes and In Situ Hybridization

Specificity of the riboprobes for MCP-1, CXCR4, and CCR2B was previously described in detail.[19,20,22,23] Complementary DNA for CCR2B was provided by Dr I.F. Charo (Gladstone Institute of Cardiovascular Disease, San Fran-

cisco, CA).[24] Complementary DNA for CXCR4 was obtained through the Acquired Immunodeficiency Syndrome (AIDS) Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). It was originally provided by Dr N.R. Landau (Salk Institute for Biological Studies, La Jolla, CA).[25]

In situ hybridization was performed as previously described.[19,20,23] In brief, after deparaffinization and rehydration, the tissue was digested in 5 μg/mL of proteinase K type XI (Sigma, St Louis, MO) for 30 minutes at 37°C. Slides were rinsed, dehydrated, and air dried. After prehybridization in 100 μL of buffer containing 0.3 mol/L of NaCl, 20 mmol/L of Tris (pH 8.0), 5 mmol/L of EDTA, 1 × Denhard's solution, 10% dextran sulfate, 10 mmol/L of DDT at 50°C, hybridization with 500 to 700,000 cpm of sulfur 35–labeled riboprobe was performed overnight. Slides were rinsed and treated with 20 μg/mL of RNase A type IIA (Sigma) for 30 minutes at 37°C. Three high-stringency washes in 0.1 × SSC (including 0.5% Tween; Sigma) at 50°C for 40 minutes each were followed by dehydration in a graded ethanol series including 0.3 mol/L of ammonium acetate. Slides were dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C between 2 and 8 weeks. Slides were counterstained with hematoxylin and eosin, dehydrated, and cover slipped with Histomount (National Diagnostics, Atlanta, GA). Hybridization of replicate tissue sections with sense probes was performed as a control.

Immunohistochemistry

Specificity and sensitivity of the antibodies against human CD3-positive T cells (rabbit antihuman; A0452; Dako, Carpenteria, CA),[18] human CD68-positive macrophages (monoclonal mouse antihuman; clone PG-M1; Dako),[18] human CCR5 (MC5),[18,26] and DARC (2C3)[21] have been reported previously for their immunohistochemical use in formal-fixed paraffin-embedded tissue. Antigen retrieval was performed on deparaffinized and rehydrated slides by steam cooking in Antigen Unmasking Solution (Vector, Burlingame, CA). Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide and biotin using the Avidin/Biotin Blocking Kit (Vector). The primary antibodies were applied for 1 hour or overnight, diluted in phosphate-buffered saline containing 1% bovine serum albumin (Sigma). After subsequent washing in phosphate-buffered saline, the tissue was incubated with the biotinylated secondary antibody for 30 minutes in a dilution of 1:500 (goat antirabbit, horse antimouse; Vector). For signal amplification, the ABC-Elite reagent (Vector) was used. 3,3'-Diaminobenzidine with nickel enhancement, resulting in a black product, was used as chromogen. Slides were counterstained with methyl green, dehydrated, and cover slipped.

The interstitial infiltrates of all specimens were scored semiquantitatively as follows: no interstitial infiltration, 0; mild, 1; moderate, 2; and severe, 3. The score reflects the overall amount of positive cells in the tubulointerstitium (including tubular epithelium, peritubular infiltrates, and vascular structures). Glomerular cells positive by immunohistochemistry (CD3, CD68, and CCR5) were classified as follows: no positive cells, 0; up to two positive cells per glomerulus, 1; up to four positive cells per glomerulus, 2; and five or more positive cells per glomerulus, 3. The numbers are given as mean scores and SEM.

RESULTS

Expression of CC Chemokines, CC Chemokine Receptors by RPA, and Their Morphological Correlation

RPA is a sensitive and specific method that allows the quantification of different mRNAs relative to housekeeping genes, but it does not identify the cellular source of expression. Total RNA was extracted from four transplant nephrectomy specimens and a normal part of a tumor nephrectomy specimen for RPA with templates for CC chemokines and CC chemokine receptors (Figs 1 and 2). The morphological features of the specimen in lane A (Figs 1 and 2) are shown in Fig 3. In a normal area of a tumor nephrectomy specimen, we were able to detect mRNA bands for interleukin-8, MCP-1, and RANTES, as well as weaker bands for lymphotactin, IP-10, and MIP-1β by RPA. This correlated with a small number of leukocyte infiltrates in this specimen (Fig 3B).

The transplant nephrectomy specimens were from grafts with acute cellular rejection (lanes C and D, Figs 1 and 2) and acute vascular rejection (lanes B and E, Figs 1 and 2). The amount of RNA of the control, reflected by the housekeeper genes (L32 and GAPDH), was similar to or greater than the amount of RNA of the transplant nephrectomy specimens. During allograft rejection, mRNA bands of lymphotactin (three of four specimens), IP-10 (four of four specimens), RANTES (three of four specimens), MIP-1α (three of four specimens), and MIP-1β (four of four specimens) were stronger in the transplant nephrectomy specimens than controls. MCP-1 mRNA expression was found in both allografts and controls (Fig 1). This was caused by expression of MCP-1 mRNA at focal sites of cellular infiltrates in the tumor nephrectomy specimens. In these areas, MCP-1 mRNA was expressed by infiltrating cells and tubular epithelium (Fig 3C), but MCP-1 mRNA expression was rare in areas of well-preserved renal tissue.

During allograft rejection, upregulation of CCR5 (three of four specimens) was present by RPA. Figure 3E and F shows the high number and morphological distribution of CD3-positive T cells and CCR5-positive cells in the intersti-
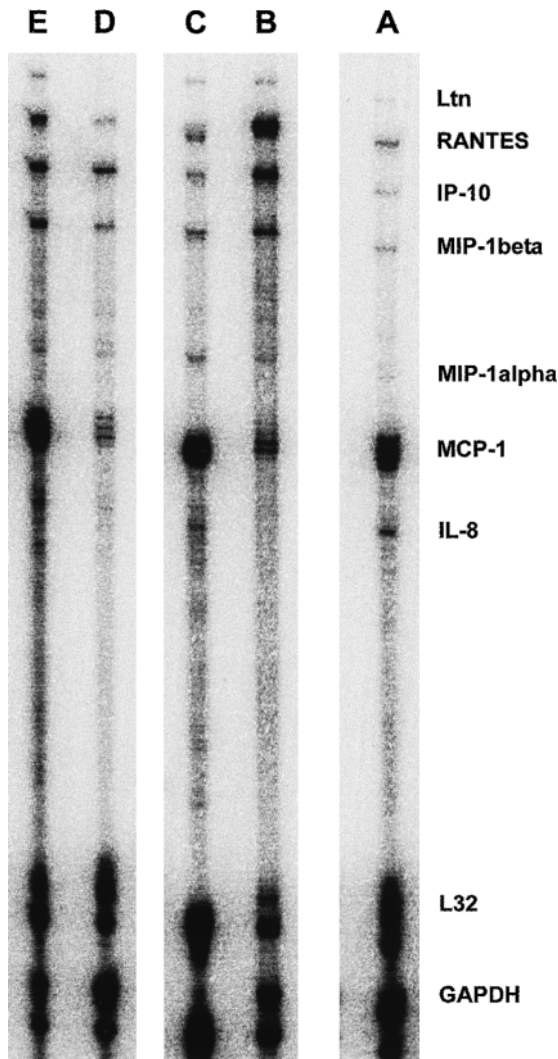
Fig 1. RPA performed with a template for human chemokines. The lanes were loaded with whole RNA extracted from (lane A) the normal area of a tumor nephrectomy specimen and (lanes B through E) transplant nephrectomy specimens. The transplant nephrectomy specimens were from patients with (lanes C and D) acute cellular rejection and (lanes B and E) acute vascular rejection. Note induction of RANTES, MIP-1β, MIP-1α, lymphotactin, and IP-10 mRNA. Abbreviations: Ltn, lymphotactin; IL-8, interleukin-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tium in the specimen used in lane B. Expression of the chemokine receptor CCR2 (four of four specimens) was also increased during transplant rejection. An increase in CCR2A mRNA was present in two cases, in addition to CCR2B mRNA. Both allografts and controls contained a strong band for CCR1 without further particular induction during allograft rejection (Fig 2).

Weaker bands of CCR4 mRNA were found in all five specimens, with a slight increase in one case with vascular rejection. None of the specimens contained detectable CCR3 and CCR8 mRNA.

Morphological Distribution of Chemokine-Receptor Expression in Normal Areas of Tumor Nephrectomy Specimens

The renal parenchyma of five tumor nephrectomy specimens was generally well preserved (Fig 3A and D), but all specimens contained small foci of leukocytes (Fig 3B). These infil-
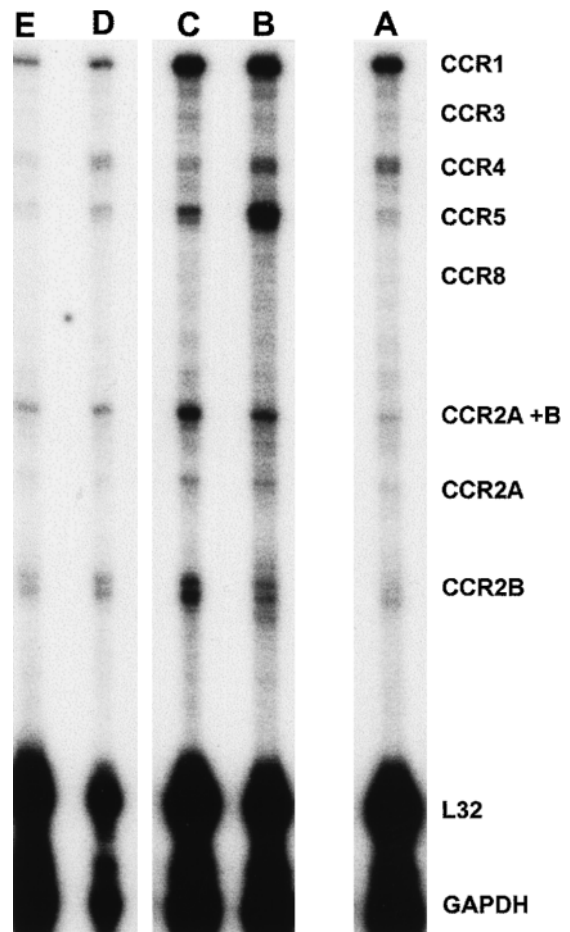

Fig 2. RPA with a template for human chemokine receptors. The lanes were loaded with whole RNA extracted from (lane A) the normal area of a tumor nephrectomy specimen and (lanes B through E) transplant nephrectomy specimens, corresponding to Fig 1. Note strong CCR1 mRNA expression in all specimens. CCR5 and CCR2 mRNA levels are increased during transplant rejection. CCR3 and CCR8 mRNA were not detectable. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
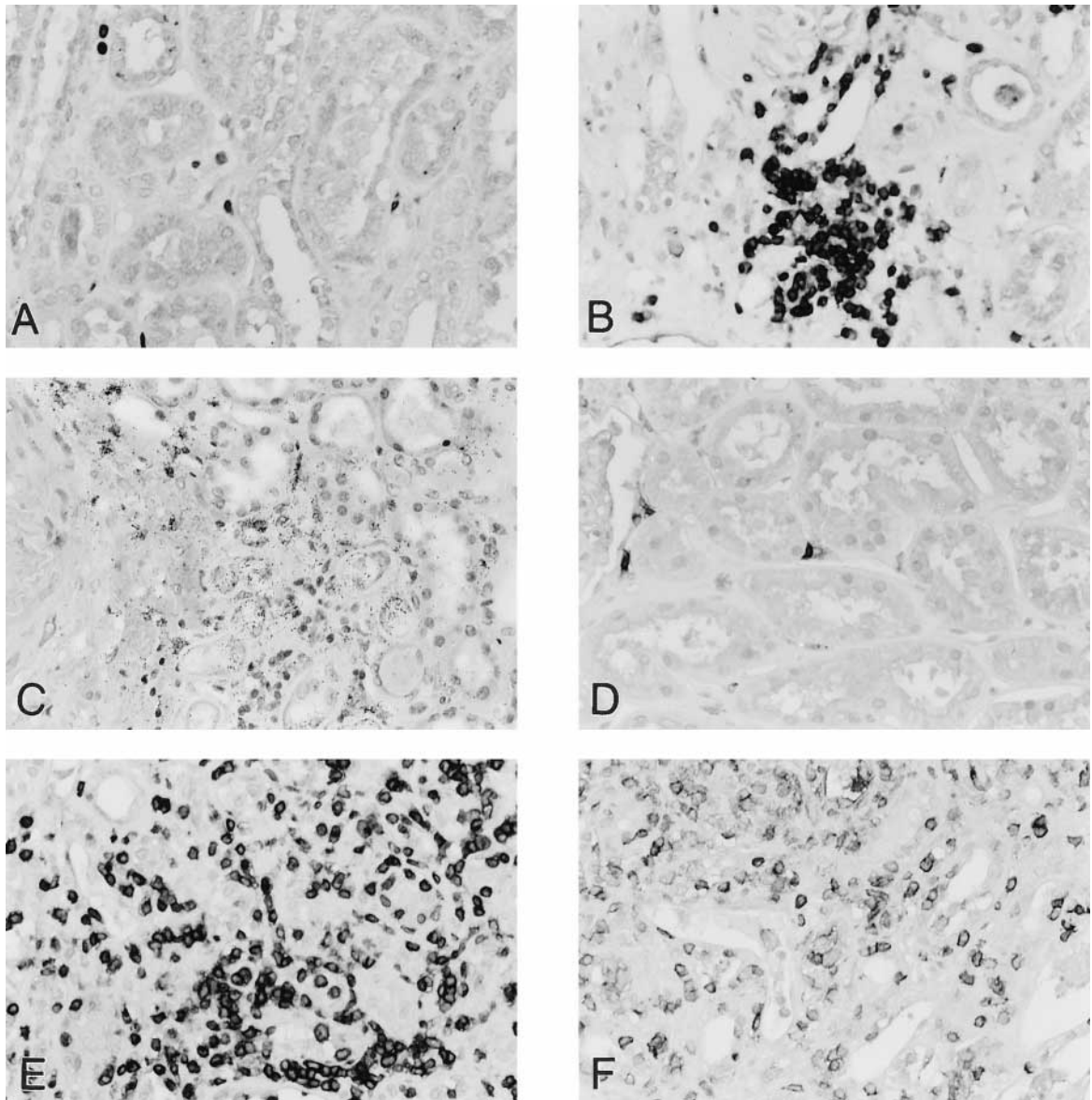
Fig 3.   Distribution of CD3-positive T cells, CCR5-positive cells, and MCP-1 expression in tumor and transplant nephrectomy specimens. (A, B, and D) Immunohistochemistry for CD3-positive T cells and CCR5-positive cells in a tumor nephrectomy specimen shows (A) the low number of T cells and (D) CCR5-positive cells in areas of well-preserved tubular interstitium and (B) small focal T-cell infiltrate in a tumor nephrectomy specimen. (C) In situ hybridization using an antisense riboprobe against MCP-1 in a tumor nephrectomy specimen. MCP-1 mRNA is expressed by infiltrating cells and tubular epithelium (compare with lane A in Figs 1 and 2). (E and F) Immunohisto-chemistry identifies (E) CD3-positive T cells and (F) CCR5-positive cells in a transplant nephrectomy specimen with vascular and cellular rejection. Note the strong diffuse interstitial infiltration by T cells and CCR5-positive cells (compare with lane B in Figs 1 and 2). (Original magnification ×400; signal, [A and B] black color, [C] deposition of silver grains.)

trates were found adjacent to globally sclerosed glomeruli, which are common in aging kidneys, and in areas of mild interstitial fibrosis (Fig 3B). The score for interstitial CD68-positive macro-phages in controls was higher (1.6 ± 0.2) than the score for interstitial CD3-positive T cells (1 ± 0; Table 1). Glomerular scores did not differ for these cell types. In areas of well-preserved renal tissue, MCP-1 expression was rare, and CXCR4- and CCR5-positive cells were only

Table 1. Interstitial Scores for Different Histological Parameters in Normal Areas of Tumor Nephrectomy Specimens and Specimens With Acute Cellular and Acute Vascular Rejection

Cell Type	Control (tumor nephrectomies)	Cellular Rejection	Vascular Rejection
CD3	1.0 ± 0.0 (n = 5)	2.2 ± 0.19 (n = 14)	2.8 ± 0.11 (n = 12)
CD68	1.6 ± 0.24 (n = 5)	2.3 ± 0.13 (n = 14)	2.8 ± 0.13 (n = 12)
CXCR4	1.3 ± 0.25 (n = 4)	2.3 ± 0.15 (n = 10)	2.5 ± 0.16 (n = 11)
CCR5	1.0 ± 0.0 (n = 5)	2.0 ± 0.2 (n = 14)	2.4 ± 0.2 (n = 12)
CCR2	1.0 ± 0.0 (n = 3)	2.0 ± 0.26 (n = 10)	2.0 ± 0.21 (n = 10)
MCP-1	2.0 ± 0.4 (n = 5)	1.8 ± 0.2 (n = 12)	2.6 ± 0.1 (n = 12)
DARC	1.2 ± 0.2 (n = 5)	2.1 ± 0.16 (n = 14)	2.6 ± 0.15 (n = 12)

NOTE. Values expressed as mean ± SEM. See Methods for details.

occasionally seen within glomerular capillaries or the interstitium (Fig 3D). DARC reactivity was found on a low number of interstitial capillaries and veins in well-preserved renal tissue (Fig 4A). In areas of interstitial infiltrates, clusters of DARC-positive vessels were found.

Morphological Distribution of Chemokine-Receptor Expression During Renal Allograft Rejection

Leukocytic allograft infiltrates were separated into four sites: (1) diffuse interstitial infiltrates, identified as accumulation of leukocytes between tubules and infiltrating the tubular epithelium (Fig 5); (2) distinct nodular aggregates, which sometimes formed follicles (Fig 6); (3) involved arteries, showing vascular rejection (Fig 7); and (4) glomeruli. Table 2 lists a descriptive summary of morphological data.

The diffuse infiltrates between tubules contained a similar number of CD3-positive T cells and CD68-positive macrophages in morphologically similar patterns of distribution (Fig 5A and B). CD3-positive T cells formed a population of small round cells without processes, whereas CD68-positive cells were larger, with a granular cytoplasmic staining, and sometimes showed processes. There was a large amount of CD68-positive material not adjacent to nuclei, ie, cross-sections of cell processes. Both cell types infiltrated the tubular epithelium. Within tubular lumina, round CD68-positive cells could commonly be detected, whereas intraluminal CD3-positive T cells were rare. CCR5-positive cells formed a major part of the diffuse interstitial infiltrates. These cells, most of them small and round without processes, commonly infiltrated the tubular epithelium but were rare within the tubular lumina. CXCR4-positive cells showed a distribution pattern indistinguishable from that of CCR5-positive cells in diffuse interstitial infiltrates, but their relative number was higher (Fig
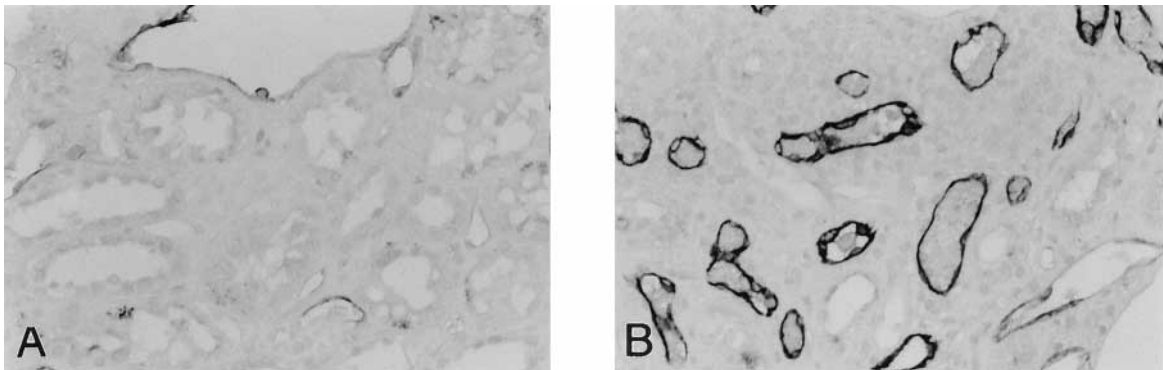

Fig 4. Immunohistochemistry for DARC in a normal area of (A) a tumor nephrectomy specimen and (B) a transplant nephrectomy specimen. During acute allograft rejection, a high number of DARC-positive capillaries can be detected in the (B) inflamed interstitium compared with (A) the tumor nephrectomy specimen. (Original magnification ×400.)
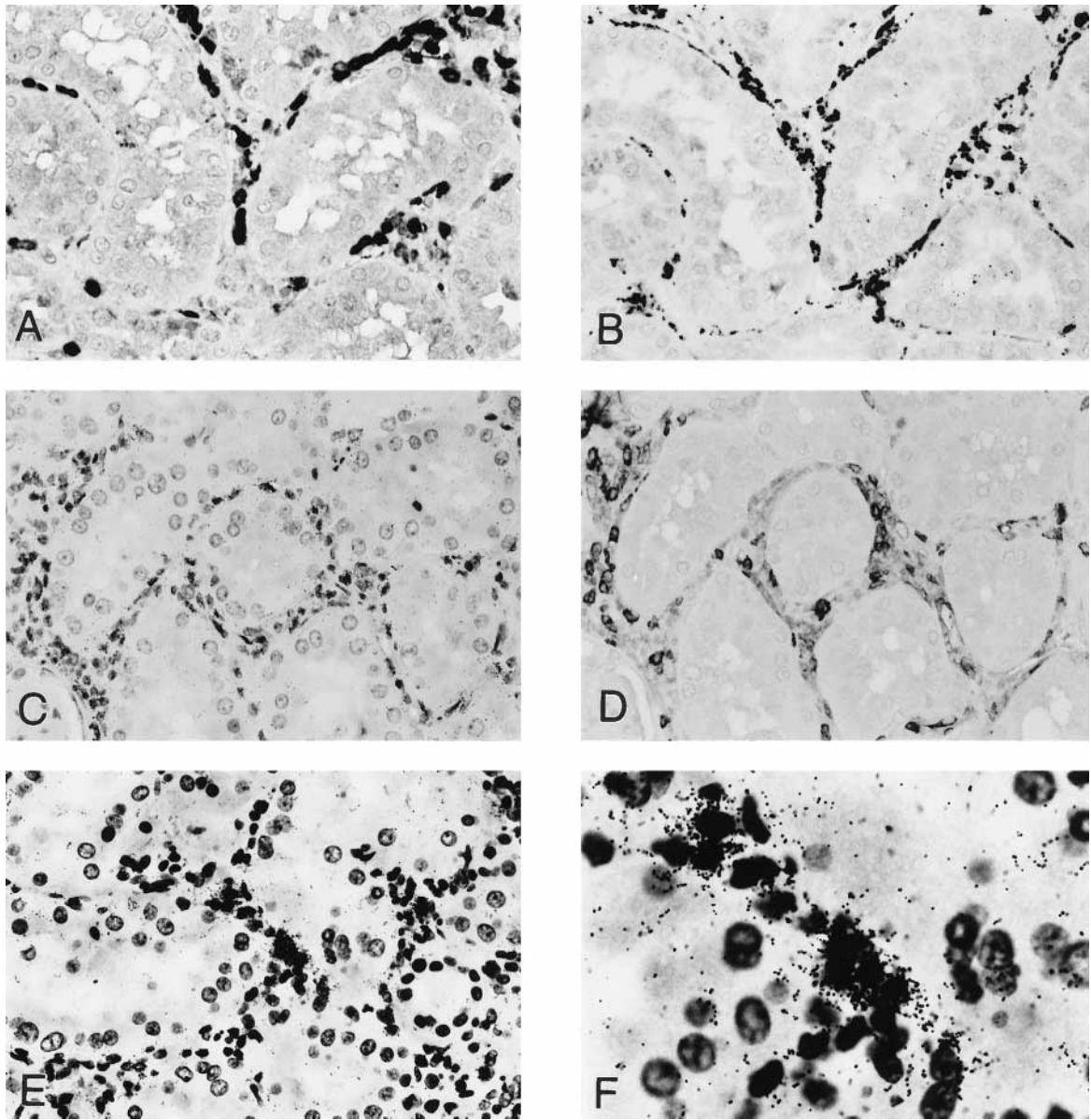
Fig 5.   Distribution of T cells, macrophages, and CCRs in diffuse infiltrates. (A and B) Immunohistochemistry for (A) CD3-positive T cells and (B) CD68-positive macrophages in serial sections of a transplant nephrectomy specimen shows the corresponding distribution of both types of infiltrating cells. (C and D) In situ hybridization using (C) an antisense riboprobe for CXCR4 and (D) immunohistochemistry for CCR5-positive cells on serial sections of a transplant nephrectomy specimen show the corresponding distribution of cells expressing these receptors in diffuse infiltrates. (E and F) In situ hybridization using an antisense riboprobe for CCR2B. (Original magnification [A-E] ×400 and [F] ×1,000.)

5C and D). CCR2B mRNA was expressed by infiltrating leukocytes, but the number was lower relative to the number of CCR5- and CXCR4-positive cells. No CCR2B mRNA expression by tubular epithelial cells was detected (Fig 5E and F). MCP-1 mRNA was expressed by intrinsic

renal cells (tubular epithelium and parietal epithelium), as well as by infiltrating cells. DARC expression was found on the endothelium of capillaries and veins. Glomerular endothelium was negative for DARC. The number of DARC-positive peritubular capillaries and veins in-
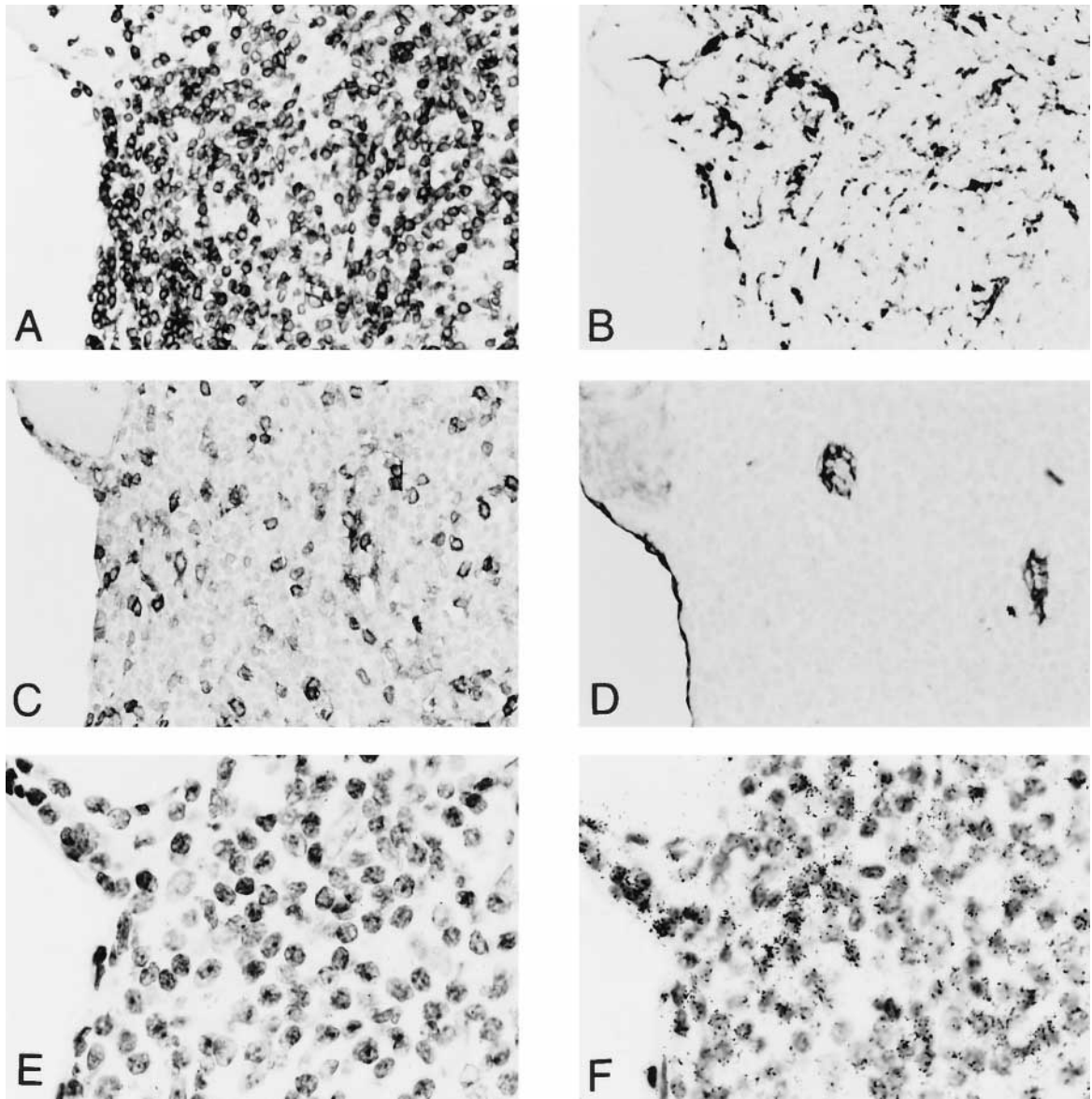
Fig 6. Distribution of cell types in nodular infiltrates. (A and B) Immunohistochemistry for (A) CD3-positive T cells and (B) CD68-positive macrophages in serial sections of a transplant nephrectomy specimen. The nodular infiltrate is located adjacent to (left) a large vein and mainly consists of CD3-positive T cells. (C) Immunohistochemistry for CCR5 on a consecutive section of the specimen shown in A. CCR5-positive cells form a relatively small part of the infiltrate. (D) Immunohistochemistry for DARC on a consecutive section of the specimen shown in (A). The adjacent large vein is covered by a DARC-positive flat endothelium, whereas two capillaries within the infiltrate show a DARC-positive high endothelium. (E and F) In situ hybridization using (E) a sense and (F) an antisense riboprobe for CXCR4 showing (E) the low number of unspecific deposited silver grains in the control. A high number of silver grains are deposited over the largest part of the infiltrating cells, resembling an infiltrate mainly consisting of CXCR4-positive cells. (Original magnification [A-D] ×400 and [E and F] ×1,000.)

creased in areas of diffuse interstitial infiltrates compared with normal areas of tumor nephrectomy specimens (Fig 4B).

Some rejecting allografts focally contained nodular aggregates of infiltrating leukocytes, mainly composed of CD3-positive T cells (Fig 6A). Within these aggregates, a smaller number of macrophages was dispersed between the T
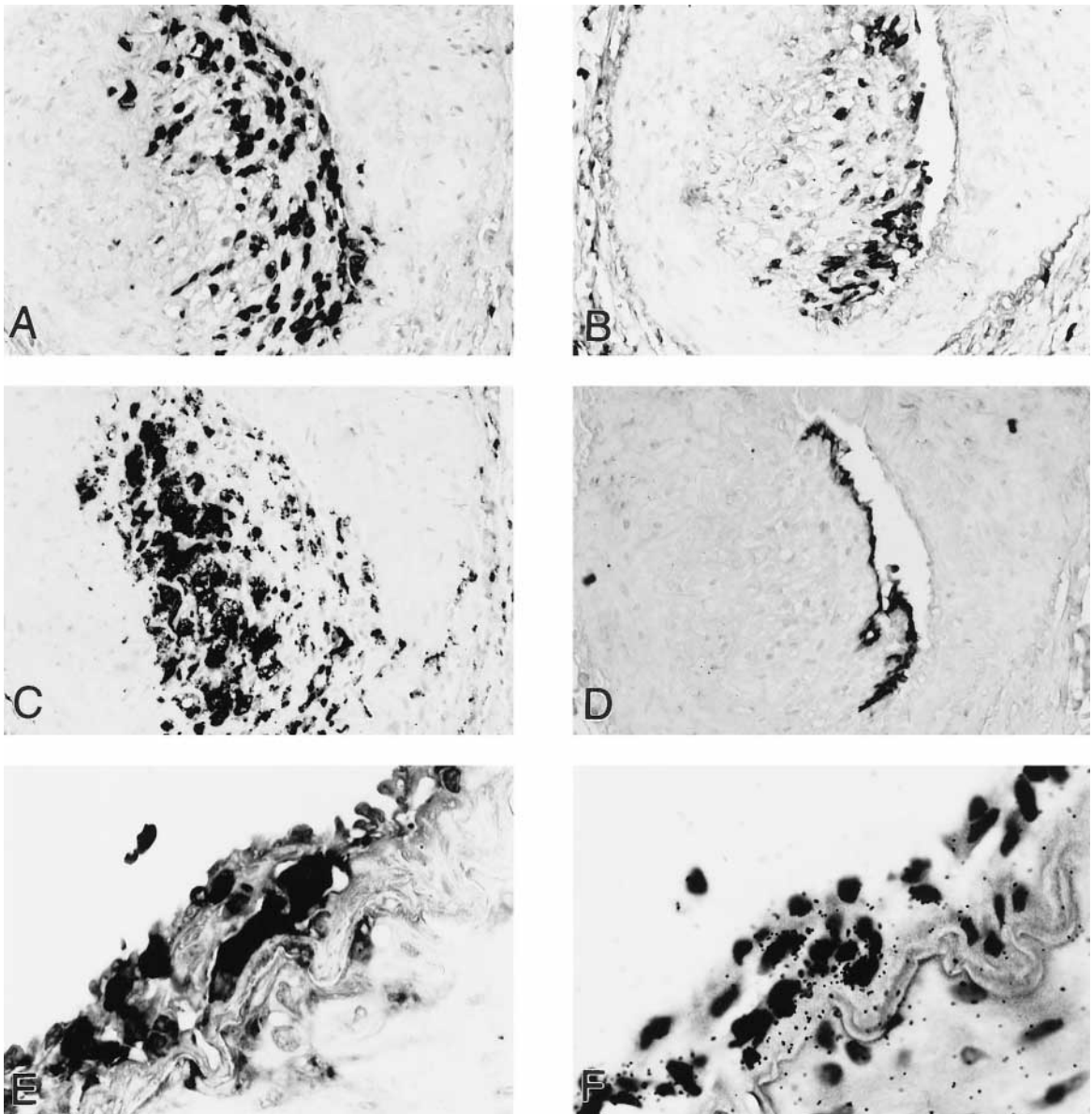
Fig 7.   Cellular infiltration during vascular rejection. (A through D) Immunohistochemistry for (A) CD3-positive T cells, (B) CCR5-positive cells, (C) CD68-positive macrophages, and (D) DARC on consecutive sections of a transplant biopsy specimen with vascular rejection. CD3-positive cells are located in the superficial subendothelial area. CCR5-positive cells follow the same distribution, but the number is less than the number of T cells. CD68-positive macrophages form a layer below the CD3-positive T cells. At this site, CCR5-positive cells were rare. (D) The rare DARC expression on arterial endothelium. Note that the lumen of the artery is completely occluded in the sections stained for CD68 and CD3. (E and F) Immunohistochemistry for (E) CCR5 and in situ hybridization using an antisense riboprobe for CXCR4 in serial sections of a transplant nephrectomy specimen with vascular rejection. CCR5- and CXCR4-positive cells can be detected at similar sites infiltrating the subendothelial intima. (Original magnification [A-D] ×400 and [E and F] ×1,000.)

cells (Fig 6B). CCR5-positive cells comprised only a small proportion of the cells within these aggregates (Fig 6C). Conversely, CXCR4 mRNA was widely expressed by cells within these aggre-

gates, indicating that most of these T cells were CXCR4 positive (Fig 6F). Cells expressing CCR2B and MCP-1 mRNA showed a distribution similar to that of CD68-positive macro-

Table 2. Descriptive Summary of Morphological Data

Antigen	Expressing Cell Type	Interstitial Infiltrates		Arteries	Glomeruli
Controls (tumor nephrectomy specimens)					
CD3	T cells	+ focal		−	+
CD68	Macrophages	++ focal		−	+
CXCR4	Inf leukocytes	+ focal		−	+
CCR5	Inf leukocytes	+ focal		−	+
CCR2	Inf leukocytes	+ focal		−	+
MCP-1	Inf leukocytes tubular epithelium	+ focal		−	+
DARC	Capillary, venous endothelium	+		−	−
		Diffuse	Nodular		
Cellular rejection					
CD3	T cells	+++	+++	−	+
CD68	Macrophages	+++	+	−	+
CXCR4	Inf leukocytes	+++	+++	−	+
CCR5	Inf leukocytes	++	+	−	+
CCR2	Inf leukocytes	+	+	−	+
MCP-1	Inf leukocytes tubular epithelium, parietal epithelium	++	+	−	+
DARC	Capillary, venous endothelium	++	++	−	−
		Diffuse	Nodular		
Vascular rejection					
CD3	T cells	+++	+++	+++	+
CD68	Macrophages	+++	+	+++	++
CXCR4	Inf leukocytes	+++	+++	+++	+
CCR5	Inf leukocytes	++	+	++	+
CCR2	Inf leukocytes	+	+	+	+
MCP-1	Inf leukocytes tubular epithelium, parietal epithelium	++	+	+	+
DARC	Capillary, venous, arterial endothelium	++	++	+ rare	−

Abbreviation: Inf, infiltrating.

phages. In nodular aggregates, the number of CCR2B-positive cells was greater than the number of CCR5-positive cells. Within aggregates, DARC-positive capillaries were commonly lined by a high endothelium (Fig 6D). The adjacent larger veins were usually DARC positive and lined by a flat endothelium.

During vascular rejection, CD3-positive T cells and CD68-positive macrophages were found in the subendothelial intima of arteries and in lower numbers throughout the vessel wall (Fig 7A and C). Usually, CD68-positive macrophages slightly outnumbered CD3-positive T cells. The number and distribution of CCR5-positive cells mirrored the number of T cells (Fig 7A and B). CXCR4-positive cells were found in a higher number than CCR5-positive cells (Fig 7F). The number of CCR2B-positive cells was lower, but they formed a significant part of the infiltrating cells in arte-

rial walls. Expression of MCP-1 mRNA by arterial endothelium was rarely found, but was common in infiltrating cells in the arterial wall. DARC expression was usually absent on arterial endothelium; only 2 of 12 artery specimens with vascular rejection showed expression by arterial endothelium. One of these arteries was completely occluded on consecutive sections (Fig 7C and D).

Macrophages outnumbered T cells in glomeruli. The cellular score for glomerular macrophages was slightly higher in vascular rejection (2.5 ± 0.21) compared with cellular rejection (1.9 ± 0.26) and tumor nephrectomy specimens (1.8 ± 0.32). Numbers of glomerular T cells and CCR5-positive cells were similar in all three groups. CXCR4-positive cells were more common in glomeruli than CCR5-positive cells. MCP-1 mRNA expression by cells in the glomer-

ular tuft and occasionally on parietal epithelial cells was present. No DARC expression on glomerular endothelium was detected.

The scores for CD3-positive T cells, CD68-positive macrophages, CXCR4, CCR5, CCR2B, and DARC were increased in specimens with acute transplant rejection (Table 1).

DISCUSSION

Several studies indicate a role for MCP-1 and its receptor, CCR2, during tubular injury in renal allograft rejection (reviewed in Segerer et al[14]), but no data are available about the morphological distribution of CCR2B in human allografts. We showed an induction of CCR2B and, in some cases, CCR2A during allograft rejection by RPA. This appears to be caused by an increased number of CCR2B mRNA–expressing infiltrating leukocytes, shown by in situ hybridization. The number of CCR2B-expressing cells detected by this method is less than the number of CCR5 protein–expressing cells and CXCR4 mRNA–expressing cells. The scattered distribution of CCR2B-expressing cells, especially in nodular aggregates of infiltrating cells, corresponding to the distribution of CD68-expressing cells, indicates that these cells are mainly macrophages. We found no evidence of CCR2B expression by renal parenchymal cells. Sites of MCP-1 expression corresponded to the sites where CCR2B-positive cells were localized.

According to their profile of secreted cytokines, Th cells are divided into Th1 and Th2 cells.[27] These T-cell subsets preferentially express certain cytokines and chemokine receptors. CXCR3, a receptor for IP-10, and CCR5, a receptor for RANTES, MIP-1α, and MIP-1β, are mainly expressed by Th1 cells. Th2 cells mainly express CCR3, CCR4, and CCR8.[28-32] The pattern of chemokine and chemokine-receptor activation identified by RPA in transplant nephrectomy specimens, with induction of IP-10, the CCR5 ligands, and CCR5 mRNA in combination with an absence of CCR3 and CCR8, is consistent with a Th1-type immune reaction within renal allografts. This provides new evidence in support of the importance of a Th1-type immune response during human renal allograft rejection, previously shown by the expression pattern of cytokines in isolated T-cell clones and by poly-

merase chain reaction in transplant biopsy specimens.[12,13,33]

During renal allograft rejection, the number of infiltrating CD3-positive T cells and CD68-positive macrophages increases, consistent with previous studies.[6,7,34] CD3-positive T cells were diffusely distributed in the renal interstitium and at times formed nodular aggregates, typically localized adjacent to large veins. The increased number of T cells infiltrating the renal allograft has been reported to be caused by both CD4- and CD8-positive subpopulations,[6,7,34] and a correlation between the distribution pattern and T-cell subset has been described[6,34,35] (ie, areas of T-cell aggregates consist mainly of CD4-positive T cells, whereas CD8-positive T cells diffusely infiltrate the interstitium). CD3-positive T cells and macrophages are the main cell types infiltrating the subendothelial space during vascular rejection.[3] The presence of CCR5-positive cells during renal allograft rejection, both at the mRNA and protein levels, has been previously described by our groups.[18,20] In concordance with these previous studies, we found upregulation of CCR5 mRNA by RPA. We found that CCR5-positive cells are differentially distributed in allograft rejection. CCR5-positive cells are a prominent subpopulation in diffuse leukocytic infiltrates, but comprise only a small number of the cells that form localized nodular aggregates of infiltrating cells. The distribution of T-cell subsets previously described suggests that the high number of CCR5-positive cells in diffuse infiltrates is mainly caused by expression of this receptor by CD8-positive T cells.

Increased expression of the CC chemokines, RANTES, MIP-1α, MIP-1β, and MCP-1, has been shown during renal transplant rejection by infiltrating cells, tubular epithelial cells, and endothelium.[36-39] It is striking that cells bearing the CCR5 receptor for these chemokines in tubulointerstitial infiltrates, tubulitis, and endothelialitis correspond to the distribution of the appropriate ligands.[18,36] It has been shown by others that MCP-1 expression during acute renal transplant rejection correlates with the number of infiltrating macrophages.[40] Furthermore, in that study, elevated urinary MCP-1 excretion during rejection episodes decreased after successful treatment.[40] By RPA, we confirmed the upregulation

of RANTES, MIP-1α, and MIP-1β. We saw no significant induction of MCP-1 mRNA during transplant rejection, which may have been caused by a relatively high expression of MCP-1 in control tissue.

The percentage of CCR5-positive cells in tubulointerstitial infiltrates was less than that of CXCR4-positive cells in this study. We previously described the high number of CXCR4-positive cells infiltrating transplant nephrectomy specimens.[19] Neointimal T cells, but not smooth muscle cells, expressed this receptor. Of the chemokine receptors studied, CXCR4 was the most widely expressed during transplant rejection.[19] In diffuse interstitial infiltrates and at sites of vascular rejection, there is a very similar distribution of CCR5-positive and CXCR4-positive cells. This might have two explanations: either a similar source of the ligands or a population of double-positive cells. We were not able to exclude double-positive cells because our attempts to combine in situ hybridization with immunohistochemistry for CCR5 have been unsuccessful. Currently, the expression of stromal-derived growth factor-1 (SDF-1), the only known ligand for CXCR4, during renal diseases is unknown. The high percentage of CXCR4-positive cells during transplant rejection is surprising because in vitro data indicate that CXCR4 is a main receptor on naïve T cells (CD45RA$^+$), whereas CCR5 is selectively expressed on memory T cells (CD45RO$^+$).[41,42] Further studies are needed to evaluate the expression of SDF-1 or other potential but still unidentified ligands of CXCR4 and the potential functional role of CXCR4 in transplantation. We speculate that the high number of CXCR4-positive cells and the low number of CCR5-positive leukocytes in nodular aggregates (ie, follicle-like structures) containing vessels with high endothelium could indicate a site of immune-cell recirculation and a source of naïve T cells.

DARC binds several CC and CXC chemokines, including RANTES and MCP-1. In the kidney, DARC is expressed on interstitial vessels ranging from the size of capillaries to larger veins and might be involved in the attraction and transmigration of leukocytes into the allograft.[43] We did not detect DARC expression on glomerular endothelium, which is in contrast to a study of

cryosection using a polyclonal antibody.[44] We have previously described the upregulation of DARC during cell-mediated renal injury.[21] The increased number of DARC-positive interstitial vessels, peritubular capillaries, and veins during allograft rejection was confirmed by this study. There is a strong association between sites of infiltration and DARC-positive vessels during renal inflammation. DARC-positive vessels occasionally showed the morphological appearance of high endothelial venules, as they occur in tonsils and lymph nodes, especially in follicular infiltrates. During vascular rejection, the arterial endothelium can become DARC positive. This site of DARC expression has not yet been described and appears to be rare because it was not detected in our previous study and was detected in only two specimens in this series.[21]

Current data lead to several questions for future studies. Two chemokines likely involved in the attraction of T cells, ie, IP-10 and lymphotactin, were upregulated during allograft rejection. Their distribution and the distribution of the corresponding receptors are currently unknown. Another intriguing result is the high expression of CCR1 in both normal kidney and rejecting renal allografts. CCR1 can be induced on mesangial cells stimulated with interferon-γ in vitro,[45] and a recent study described increased T-cell and macrophage infiltration caused by an enhanced Th1 response in CCR1-deficient mice during nephrotoxic serum nephritis.[46] The distribution of CCR1 in normal and allograft kidneys is currently unknown, but the predicted counterregulatory role of this receptor might be of major impact for therapeutic studies and therefore merits further evaluation.

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