

Expression of the Chemokine Monocyte Chemoattractant Protein-1 and Its Receptor Chemokine Receptor 2 in Human Crescentic Glomerulonephritis

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Abstract. Crescents are morphologic manifestations of severe glomerular injury. Several chemokines and their receptors have been demonstrated to be involved in animal models of crescentic glomerulonephritis (cGN) and are potential targets for therapeutic interventions. Therefore, the expression of monocyte chemoattractant protein-1 (MCP-1), its receptor chemokine receptor 2B (CCR2B), and CCR5 in human cGN was studied. MCP-1 and CCR2B mRNA expression was evaluated, by *in situ* hybridization, in serial sections of 23 renal biopsies from patients with cGN. T cells, macrophages, and CCR5-expressing cells were examined by immunohistochemical analysis. MCP-1 mRNA was expressed by cells in crescents, parietal epithelium, and tubular epithelium, as well as by infiltrating leukocytes in the tubulointerstitium. The expression of CCR2B mRNA was observed in cells in glomeruli and crescents and in infiltrating leukocytes in the tubulointerstitium. CCR2B mRNA expression could not be clearly localized

to intrinsic renal cells; evidence that most of the CCR2B-expressing cells were leukocytes is provided. CD3-positive T cells formed the major part of the interstitial cell infiltrates but were rare within the glomerular tufts. CD68-positive macrophages constituted a major population of infiltrating cells in crescents and contributed significantly to the interstitial infiltrates. The number of glomerular macrophages was associated with the number of MCP-1- and CCR2B-positive glomerular cells. Expression of CCR2B was significantly correlated with interstitial CD3-positive T cells. CCR5 expression was restricted to infiltrating leukocytes and was correlated quantitatively and by localization with interstitial CD3-positive T cells and CD68-positive macrophages. These first morphologic data on the distribution of CCR2-positive cells in human cGN suggest differential effects of chemokines and their receptors on the distribution of infiltrating leukocytes in different compartments of the kidney.

Crescents, defined as two or more layers of cells partially or completely filling Bowman's space, are morphologic expressions of severe inflammatory glomerular injury and indicators of poor prognoses (1). Proliferating parietal epithelium and macrophages are thought to be major contributors to the cells in crescents, but T cells and fibroblasts are also involved (2,3). The cell composition and factors that lead to the formation and resolution of crescents are currently under intensive investigation (4–6). Accumulating data in animal models and human subjects point to the importance of a cell-mediated immune response in crescentic glomerulonephritis (cGN) (for review, see reference 7). The process of leukocyte extravasation from the circulation to the site of inflammation involves a cascade of interactions between soluble

factors and surface molecules expressed by leukocytes and endothelial cells (8). The importance of chemokines and their receptors at multiple stages in this process has been demonstrated in the past decade. Chemokines are a superfamily of small chemotactic cytokines that specifically attract subsets of leukocytes (for review, see references 9–12). Chemokines function via G protein-coupled receptors, which usually bind more than one chemokine of the same subgroup. The CC chemokines monocyte chemoattractant protein-1 (MCP-1), which binds to the CC chemokine receptor 2 (CCR2), and regulated upon activation, normal T cell-expressed and secreted (RANTES), which binds to CCR5, CCR3, and CCR1, have been most extensively studied in rodent models of renal diseases (for review, see reference 9). *In vitro* data indicate that the chemokine receptors CCR2 and CCR5 are both expressed by at least some subsets of T cells and macrophages, which are the main cell types comprising the interstitial infiltrates in inflammatory renal diseases and are of major prognostic importance (13,14). The distribution of CCR2-positive cells in human renal diseases is still unknown. We previously described the expression of CCR5 by infiltrating cells in various “noncrescentic” renal diseases and demonstrated that these cells (mainly T cells) form a major part of the interstitial inflammatory cell

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infiltrates commonly observed in renal diseases (15). The characterization of chemokine receptor-expressing cells, corresponding to specific morphologically defined processes in the evolution of the diseases, can provide new insights into the pathogenesis of cGN and is a prerequisite for studies with receptor-blocking agents.

Materials and Methods

Tissue Samples

A total of 23 renal biopsies from patients with cGN were studied. Included cases were from biopsies examined during the period from 1996 to 1999 in the Department of Pathology, University of Washington (Seattle, WA). All accessioned cases for which sufficient material was available for multiple additional immunohistochemical studies after completion of routine diagnostic evaluations were included. No further selection criteria were applied. Patient samples were obtained under the general consent to have a renal biopsy performed and/or accessioned at the University of Washington. Under these conditions, which were approved by the University of Washington internal review board for human subjects, no patient identifiers may be used in studies involving the biopsy tissue; therefore, correlative functional parameters could not be obtained for this study. The specimens were fixed in 10% phosphate-buffered formalin, processed, embedded in paraffin, and sectioned at 4 μ m, using conventional techniques. Chemokine receptor-overexpressing cell lines were used as positive controls for *in situ* hybridization and Northern blotting. HOS cells overexpressing CCR1, CCR3, CCR4, CCR5, and CXCR4 chemokine receptor 4 (CXCR4) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health), to which they were originally provided by Dr. N. R. Landau (Salk Institute for Biological Studies, La Jolla, CA). CXCR4 chemokine receptor 4 (CXCR4)(16). CCR2B-overexpressing cells were kindly provided by Dr. I. F. Charo, Gladstone Institute of Cardiovascular Disease (San Francisco, CA).

Molecular Probes

The establishment of the MCP-1 probe was previously described in detail (17). The cDNA for CCR2B was kindly provided by Dr. I. F. Charo (18). For nonradioactive Northern blotting, the linearized CCR2B plasmid was transcribed into a digoxigenin-labeled riboprobe using digoxigenin-labeled UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN). The 28S probe was previously described (19,20).

In Situ Hybridization

In situ hybridization was performed as described previously (19,20). Slides were deparaffinized in xylene and rehydrated in graded ethanols. After rinsing with 0.5 \times SSC (1 \times SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), the tissue was digested for 30 min at 37°C with proteinase K type XI (5 μ g/ml; Sigma Chemical Co., St. Louis, MO) in proteinase K buffer (500 mM NaCl, 10 mM Tris, pH 8.0). Slides were rinsed three times with 0.5 \times SSC, dehydrated, and air-dried, followed by a 2-h incubation in 100 μ l of prehybridization buffer (0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM ethylenediaminetetraacetate, 1 \times Denhardt's solution, 10% dextran sulfate, 10 mM di-thiothreitol) at 50°C. Hybridization was performed at 50°C with 50 μ l of prehybridization buffer containing 500,000 to 700,000 cpm of ³⁵S-labeled riboprobe/slide. After overnight hybridization, slides were washed three times with 2 \times SSC and then treated with RNase A type

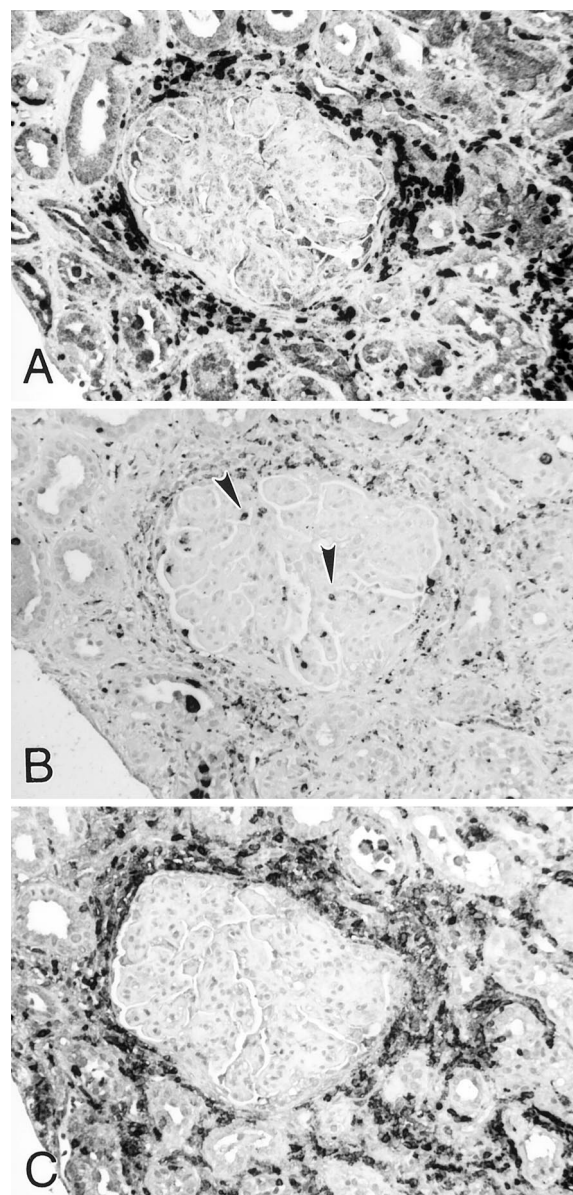


Figure 1. Distribution of T cells, macrophages, and chemokine receptor 5 (CCR5)-positive cells. (A) Immunohistochemical analysis of CD3-positive T cells, showing interstitial CD3-positive T cells (immunohistochemical signal indicated by the black product) accentuated in the periglomerular area but no positive cells in the glomerulus. Original magnification, $\times 200$. (B) Immunohistochemical analysis of CD68-positive macrophages. In addition to the interstitial infiltrate, several macrophages are evident within the glomerulus (arrowheads). Original magnification, $\times 200$. (C) Immunohistochemical analysis of CCR5-positive cells. The cell distribution mirrors that of CD3-positive T cells, with no positive cells within the glomerulus. Original magnification, $\times 200$.

IIA (20 μ g/ml; Sigma) for 30 min at 37°C. This treatment was followed by three 30-s washes at room temperature with 2 \times SSC and three high-stringency, 40-min washes at room temperature with 0.1 \times SSC (containing 0.5% Tween) at 50°C. After three washes with 2 \times SSC, slides were dehydrated in graded ethanols containing 0.3 M ammonium acetate and were air-dried. After being dipped in NTB2

Table 1. Mean \pm SEM of positive cell numbers per high-power field

	No. of Biopsies	Cells/Field		
		Interstitial	Glomerular	Overall
CD3	23	102.5 \pm 8.6	1.7 \pm 0.4	104.1 \pm 8.7
CD68	22	33.2 \pm 3.0	7.0 \pm 1.4	40.2 \pm 3.9
MCP-1	21	18.6 \pm 3.4	2.4 \pm 0.4	20.9 \pm 3.5
CCR2	17	19.7 \pm 3.3	1.4 \pm 0.3	21.1 \pm 3.3
CCR5	22	59.1 \pm 4.4	1.1 \pm 0.3	60.3 \pm 4.4

Table 2. Percentage of glomeruli with crescents including three or more positive cells

	Glomeruli (%)
CD3	22
CD68	75
MCP-1	66
CCR2	57
CCR5	39

nuclear emulsion (Kodak, Rochester, NY), slides were exposed in the dark at 4°C for 2 to 8 wk. After development, slides were counterstained with hematoxylin and eosin, dehydrated, and coverslipped with Histomount (National Diagnostics, Atlanta, GA).

Northern Analysis

Total RNA was isolated from chemokine receptor-overexpressing HOS cells using the TOTALLY RNA total RNA isolation kit (Ambion, Austin, TX), according to the instructions provided by the manufacturer. RNA samples containing 10 μ g/lane were separated by electrophoreses through a 1% agarose-formaldehyde gel. The RNA was transferred to a nylon membrane (BrightStar-Plus; Ambion) using the VacuGene XL vacuum blotting system (Pharmacia Biotech, Piscataway, NJ) and was cross-linked to the membrane with ultraviolet light. The part with the size marker was removed, rinsed with 5% acetic acid for 15 min, and stained with methylene blue (0.5 M sodium acetate, pH 5.2, 0.04% methylene blue). The membrane was placed in prewarmed hybridization buffer (NorthernMax; Ambion) for 2 h at 65°C, followed by overnight hybridization at 65°C with the digoxigenin-labeled riboprobe at a concentration of 10 ng/ml. The membrane was washed twice for 5 min at room temperature with 2 \times SSC containing 0.1% sodium dodecyl sulfate, followed by two high-stringency washes for 15 min at 65°C with 0.1 \times SSC containing 0.1% sodium dodecyl sulfate. The BrightStar BioDetect nonisotopic detection system (Ambion) was used, according to the instructions provided by the manufacturer, for development of the signal.

Immunohistochemical Analysis

The MC5 antibody against human CCR5 and its specific use for paraffin-embedded tissue were previously described (15). Antibodies to CD3-positive T cells (rabbit anti-human CD3 antibody A0452; Dako, Carpinteria, CA) and CD68-positive macrophages (mouse monoclonal anti-human CD68 antibody, clone PG-M1; Dako) were used for immunohistochemical analyses, similar to previously described protocols (15,21). Antigen retrieval was performed on deparaffinized and rehydrated slides, by steam-heating in antigen-unmask-

ing solution (Vector, Burlingame, CA). Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. The avidin/biotin blocking kit (Vector) was used to block endogenous biotin. The primary antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin (Sigma) and were applied for 1 h. After subsequent washes with phosphate-buffered saline, the tissues were incubated with biotinylated secondary antibodies for 30 min (goat anti-rabbit Ig and horse anti-mouse Ig; Vector). The ABC-Elite reagent (Vector) was used for signal amplification. Diaminobenzidine was applied as a chromogen, and slides were counterstained with methyl green, dehydrated, and coverslipped.

Ten high-power fields (magnification, $\times 400$), encompassing an area of 0.189 mm² each, were evaluated for each biopsy. Individual positive cells were counted in the interstitium and glomeruli. A cell was considered positive by *in situ* hybridization when more than four silver grains were concentrated above or immediately adjacent to a nuclear profile. Because of the somewhat punctate, cytoplasmic staining pattern of CD68-positive cells, quantitative evaluation was difficult, especially in the tubulointerstitium. To minimize this problem, we counted the color reaction product as indicating a positive cell only when it was associated with a nucleus. This approach introduces the potential problem of underestimation of the number of interstitial macrophages.

Statistical Analyses

Results are given as means \pm SEM. The Wilcoxon test was used for the comparison of mean values. Evaluations of the correlations were performed using the Spearman correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

CD3-Positive T Cells Form a Major Part of Interstitial Infiltrates but Are Rare in Glomeruli

A prominent feature of all cases was infiltration of the tubulointerstitium by mononuclear leukocytes (Figure 1, A and B). The mononuclear cell infiltrates were often accentuated around glomeruli (Figure 1A). CD3-positive T cells formed a major part of the interstitial infiltrates (Figure 1A; Table 1). Infiltration of the tubular epithelium by CD3-positive T cells was regularly observed and consisted of up to four cells/tubular cross-section. Small, nodular, interstitial infiltrates of mononuclear leukocytes consisted mainly of T cells.

In contrast to the large number of tubulointerstitial CD3-positive T cells, the number of T cells observed within glomeruli was small. Only 1.6% of the total number of T cells were localized in glomerular tufts or within crescents. Bowman's capsule, which normally separates the glomerular space

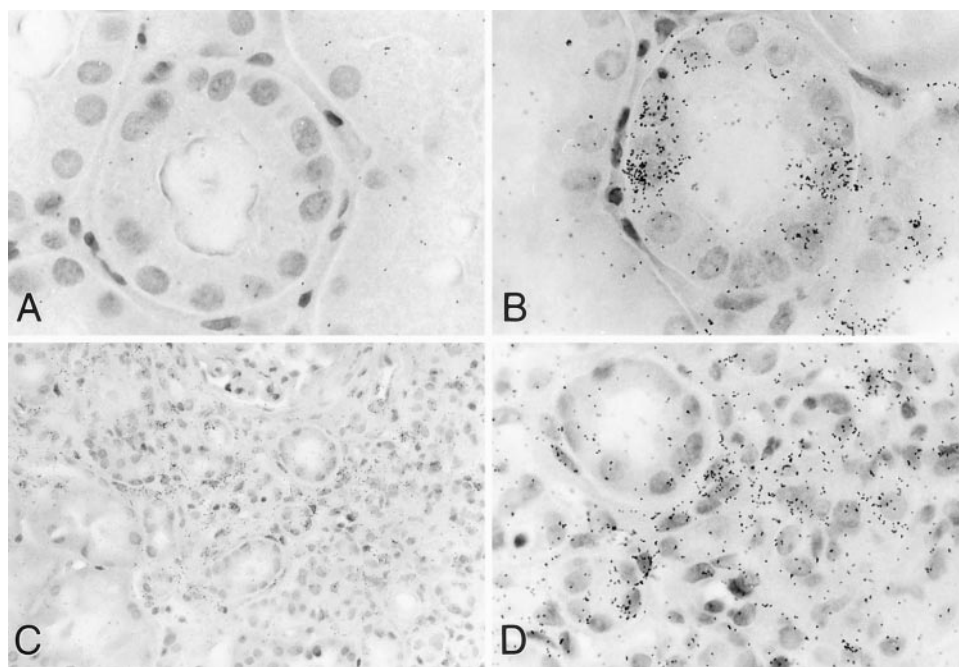


Figure 2. Monocyte chemoattractant protein-1 (MCP-1) mRNA expression in the tubulointerstitium. (A) *In situ* hybridization using a MCP-1 sense riboprobe, demonstrating a small number of nonspecifically deposited silver grains. Original magnification, $\times 1000$. (B) *In situ* hybridization using a MCP-1 antisense riboprobe, demonstrating MCP-1 mRNA expression by tubular epithelial cells (*in situ* signal indicated by the deposition of silver grains). Original magnification, $\times 1000$. (C and D) *In situ* hybridization using a MCP-1 antisense riboprobe, demonstrating MCP-1 mRNA expression by infiltrating interstitial cells. Original magnification: $\times 400$ in C; $\times 1000$ in D.

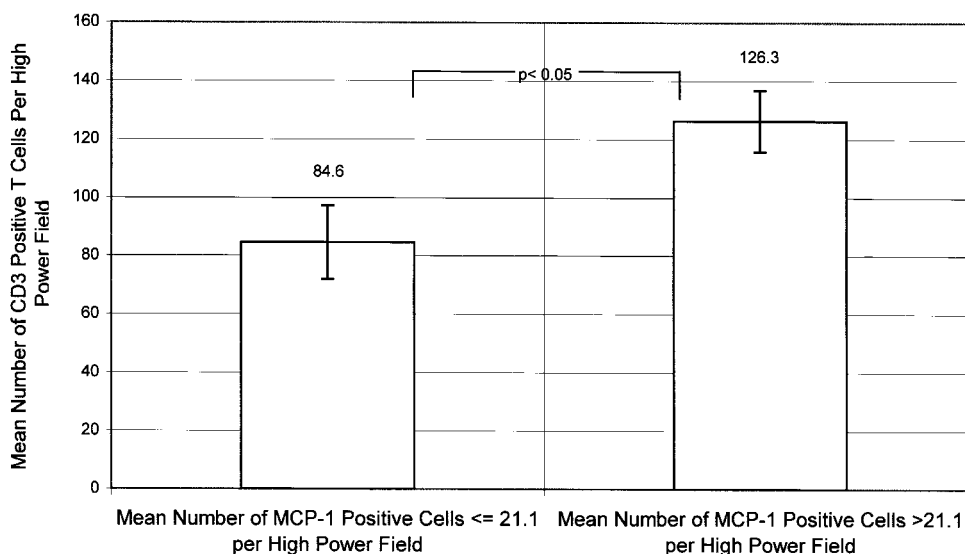


Figure 3. Comparison of the mean numbers of CD3-positive T cells in two groups, with smaller (left) or larger (right) numbers of MCP-1-expressing cells ($P < 0.05$). Groups were formed according to the median of the mean number of MCP-1-positive cells.

from the interstitium, was usually destroyed in the subset of crescents with larger numbers of CD3-positive T cells.

CD68-Positive Macrophages Are the Main Infiltrating Cells in Glomeruli and Crescents

CD68-positive macrophages were common in the tubulointerstitial infiltrates (Figure 1B). As discussed above, the number of interstitial macrophages might be biased by the staining

pattern; therefore, the absolute numbers of T cells and macrophages cannot reliably be compared. Although the distributions of T cells and macrophages in the interstitium were often similar, we observed no numerical correlation between the mean number of CD3-positive T cells and the mean number of CD68-positive macrophages (data not shown). CD68-positive macrophages were commonly observed within the tubular lumina.

In addition to comprising a large percentage of the intersti-

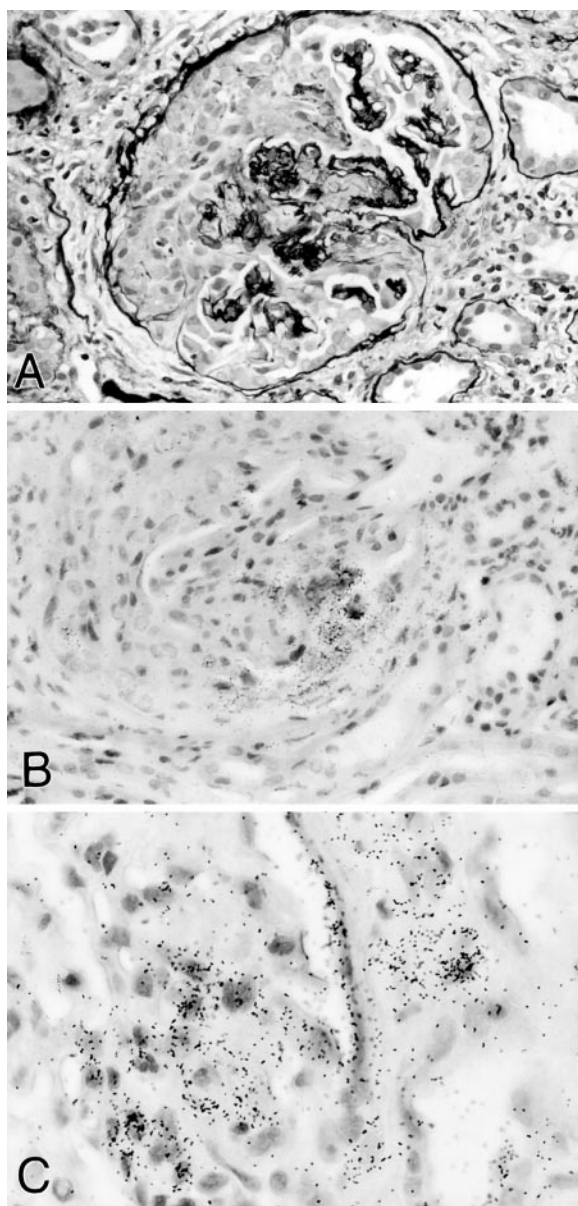


Figure 4. Expression of MCP-1 mRNA in cells of the glomerular tuft, cells of crescents, and Bowman's capsule epithelium. (A) Glomerulus with a cellular crescent (silver-methenamine staining). Original magnification, $\times 400$. (B) *In situ* hybridization using a MCP-1 antisense riboprobe, demonstrating MCP-1 mRNA expression in cells of the remaining tuft and cells of the cellular crescent surrounding the tuft. Original magnification, $\times 400$. (C) *In situ* hybridization using a MCP-1 antisense riboprobe, demonstrating MCP-1 mRNA expression by parietal epithelial cells and cells within the glomerular tuft. Original magnification, $\times 1000$.

tial infiltrates, CD68-positive macrophages were the main infiltrating cell type in crescents and glomerular tufts (Figure 1B). The glomerular area contained a mean of 7.0 CD68-positive macrophages/high-power field and 17.5% of the total number of macrophages. In comparison, only 1.6% of the total number of CD3-positive T cells were observed in glomeruli.

Seventy-five percent of glomeruli with crescents contained three or more macrophages (compared with 22% with three or more CD3-positive T cells) (Table 2).

Tubular Epithelial Cells, Interstitial Infiltrating Cells, and Cells in the Glomerular Tufts and in Crescents Express MCP-1

Representative examples from biopsies hybridized with the MCP-1 sense and antisense riboprobes are presented in Figure 2, A and B. Only the antisense riboprobe revealed focal hybridization, with the large number of silver grains signaling a positive reaction (Figure 2B). Sites of MCP-1 expression were associated with mononuclear cell infiltration in both the glomeruli and tubulointerstitium. In contrast, MCP-1 mRNA expression was rarely observed in areas of well preserved renal architecture.

In the tubulointerstitium, MCP-1 mRNA was expressed by tubular epithelium (morphologically, primarily of distal tubules) and infiltrating mononuclear cells (Figure 2, B to D). MCP-1 mRNA remained detectable in atrophic tubules. MCP-1 mRNA-expressing cells, similar to CD68-positive macrophages, were sometimes observed within tubular lumina. MCP-1-positive cells were rare in areas of marked nodular accumulation of mononuclear leukocytes. Biopsies containing larger numbers of MCP-1 mRNA-positive cells demonstrated significantly larger numbers of infiltrating CD3-positive T cells (Figure 3).

MCP-1 mRNA was expressed by cells in the glomerular tuft, by cells in crescents, and by parietal epithelial cells (Figure 4, B and C). Sixty-six percent of glomeruli with crescents contained three or more MCP-1-positive cells and, overall, 12% of MCP-1-positive cells were observed in glomeruli. This distribution mirrored the distribution of CD68-positive macrophages. Biopsies containing large numbers of glomerular MCP-1-positive cells displayed correspondingly larger numbers of CD68-positive cells within the glomeruli, but the difference did not reach statistical significance (Figure 5).

CCR2B mRNA Is Commonly Expressed by Infiltrating Cells in Glomeruli and in the Tubulointerstitium

The specificity of the CCR2B riboprobe was demonstrated by Northern blotting (Figure 6) and by *in situ* hybridization of transfected HOS cells overexpressing the chemokine receptors CXCR4 and CCR1 to CCR5. A single band with the expected size for CCR2B was demonstrated only in the CCR2B-overexpressing cell line by Northern blotting. No cross-hybridization was detected in RNA isolated from cells expressing CCR1, CCR3, CCR4, CCR5, or CXCR4 (Figure 6). Using the CCR2B antisense riboprobe for *in situ* hybridization, significant deposition of silver grains was detected only in pellets of CCR2B-overexpressing HOS cells (data not shown). There was no significant deposition of silver grains in tissue hybridized with the sense riboprobe, as illustrated in Figure 7.

CCR2B mRNA-expressing infiltrating mononuclear leukocytes were common in the tubulointerstitium (Figure 7, C and D). These cells were observed to be clustered around MCP-1 mRNA-positive tubular epithelium, as demonstrated by *in situ* hybridization of replicate tissue sections (Figure 8, C and D).

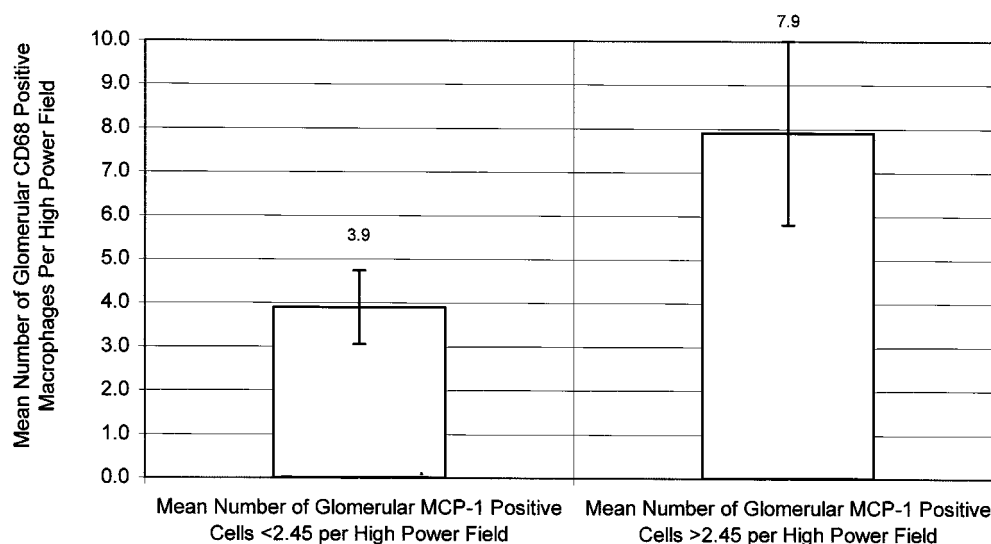


Figure 5. Comparison of the mean numbers of glomerular CD68-positive macrophages in two groups, with smaller (left) or larger (right) numbers of glomerular MCP-1-expressing cells (NS). Groups were formed according to the median of the mean number of glomerular MCP-1-positive cells.

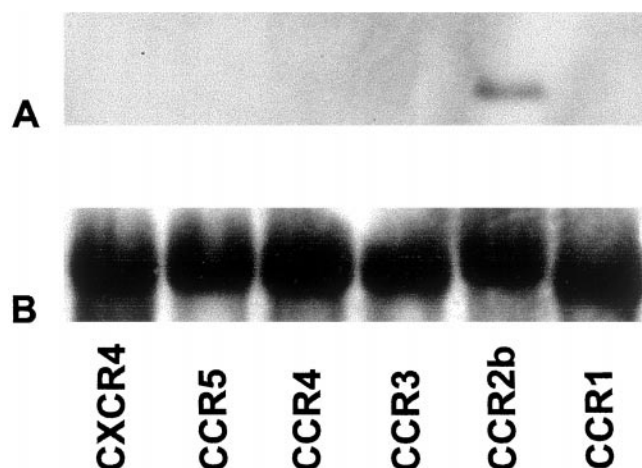


Figure 6. (A) Northern blot hybridized with a digoxigenin-labeled antisense probe directed against CCR2B, demonstrating a single band (3.5 kb) in CCR2B-overexpressing cells. (B) Hybridization with a 28S probe, demonstrating similar amounts of RNA in each lane.

Furthermore, CCR2B-positive cells were commonly arranged around crescentic glomeruli (Figure 8, A and B). There were significant correlations between the mean numbers and localization of interstitial CCR2B-positive cells and interstitial CD3-positive T cells (Figures 7, C to F, and 9). Areas with interstitial infiltrates organized as lymphoid follicles contained a few CCR2B-positive cells, which were located mainly in the periphery of the follicles. Few CCR2B mRNA-positive cells were observed within tubular lumina.

CCR2B mRNA-expressing cells commonly infiltrated glomerular tufts and crescents, but their numbers were lower than the numbers of glomerular macrophages (Figure 10; Table 2). Seven percent of the total CCR2B mRNA-expressing cells were observed in glomeruli. Biopsies with large numbers of

CD68-positive macrophages in glomeruli and crescents exhibited a trend toward larger numbers of glomerular CCR2B-expressing cells, but the correlation did not reach statistical significance ($r^2 = 0.5$, NS). No convincing CCR2B mRNA hybridization signal was observed in intrinsic renal cells, but focal expression by endothelial cells of small capillaries could not be excluded in areas with extensive interstitial CCR2-positive infiltrates.

CCR5-Positive Cells Are a Prominent Part of the Tubulointerstitial Infiltrates, and the Mean Number Is Correlated with the Number of Interstitial T Cells and Interstitial Macrophages

CCR5 expression was a prominent feature of interstitial infiltrating leukocytes (Figure 1C). These cells were commonly observed to be infiltrating the tubular epithelium. Clustering around Bowman’s capsules of inflamed glomeruli was also a common feature for CCR5-positive leukocytes. The best morphologic concordance was observed between the distributions of CD3-positive T cells and CCR5-positive cells, which led to a significant correlation between the mean number of CCR5-positive cells and the mean number of CD3-positive T cells ($P < 0.05$). Furthermore, the numerical correlation between CCR5-positive cells and interstitial CD68-positive macrophages reached statistical significance ($P < 0.05$). A morphologic correlation between areas of macrophage infiltration and CCR5-positive cells was rarely observed (Figure 11, G and H), indicating a small subset of CCR5-positive macrophages. CCR5-positive cells within tubular lumina were rare, indicating that the intratubular CD68-positive cells were mainly CCR5-negative.

Within glomeruli, CCR5-positive cells were rare but were detectable in a subset of crescents (1.9% of the CCR5-positive cells). The CCR5 staining of cells in crescents was generally

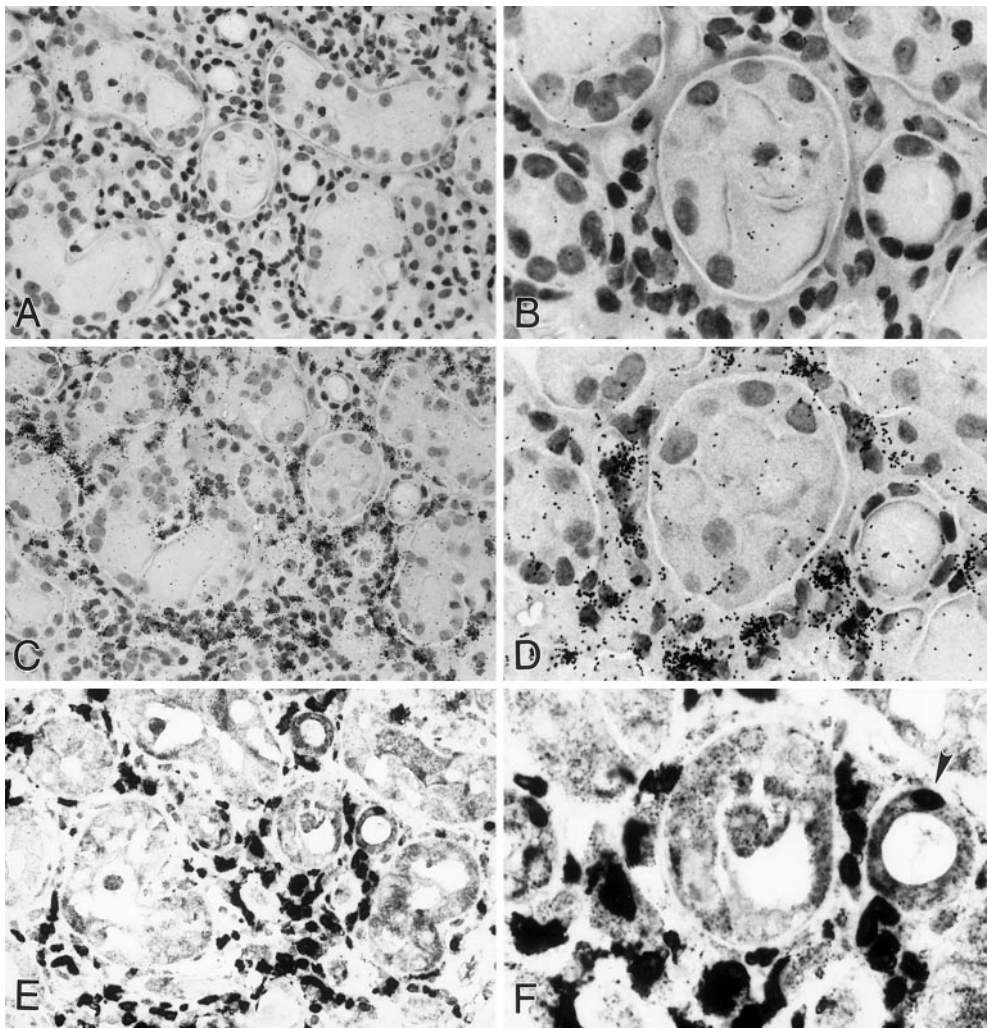


Figure 7. Distribution of T cells and CCR2B mRNA-positive cells. (A and B) *In situ* hybridization using a CCR2B sense riboprobe, demonstrating a small amount of nonspecific deposition of silver grains. Original magnification: $\times 400$ in A; $\times 1000$ in B. (C and D) *In situ* hybridization using a CCR2B antisense riboprobe, demonstrating CCR2B mRNA expression in infiltrating cells (replicate section of the biopsy shown in A). Original magnification: $\times 400$ in C; $\times 1000$ in D. (E and F) Immunohistochemical analysis of CD3-positive T cells, illustrating the distribution of peritubular T cells, which are characterized by homogeneous dark staining of the cell bodies. Note the infiltration of the tubular epithelium (arrowhead). Original magnification: $\times 400$ in E; $\times 1000$ in F.

weaker than the staining of most of the interstitial, CCR5-positive, infiltrating cells. The glomerular CD68-positive cells outnumbered the CCR5-positive cells. A glomerulus with an extraordinarily large number of infiltrating cells and CCR5-positive cells (the most encountered in this series) is illustrated in Figure 10F. No CCR5 expression was detectable in intrinsic renal cells.

Discussion

The chemokine MCP-1 and its receptor CCR2 are likely involved in the recruitment of macrophages in inflammatory renal diseases. No data on the distribution of CCR2-positive cells in human renal diseases are currently available. cGN, which is the most aggressive form of inflammatory glomerular injury, is largely the result of cell-mediated immune injury, including prominent macrophage infiltration of glomeruli (2,7). This is the first study to demonstrate the expression of

CCR2B mRNA in biopsies from patients with cGN. CCR2B mRNA was expressed by infiltrating mononuclear leukocytes, and CCR2B mRNA-expressing cells were common in inflamed glomeruli and within crescents. The numbers and distribution of glomerular CD68-positive monocytes/macrophages indicated that these cells were the main glomerular cell type that expressed CCR2B mRNA. In addition, a correlation between CCR2B mRNA-expressing interstitial cells and similarly located CD3-positive T cells indicated that some CCR2B-positive T cells infiltrated the interstitium during this disease process. *In vitro* studies indicated that monocytes/macrophages (22–24), activated T cells (25–27), activated B cells (28), dendritic cells (29), and activated natural killer cells (30,31) express CCR2. CCR2 is a receptor for the chemokines MCP-1 through -5 (18,32–34). Two isoforms of CCR2 have been described, *i.e.*, CCR2A and CCR2B. Currently, CCR2B is thought to be the major isoform expressed on cultured and

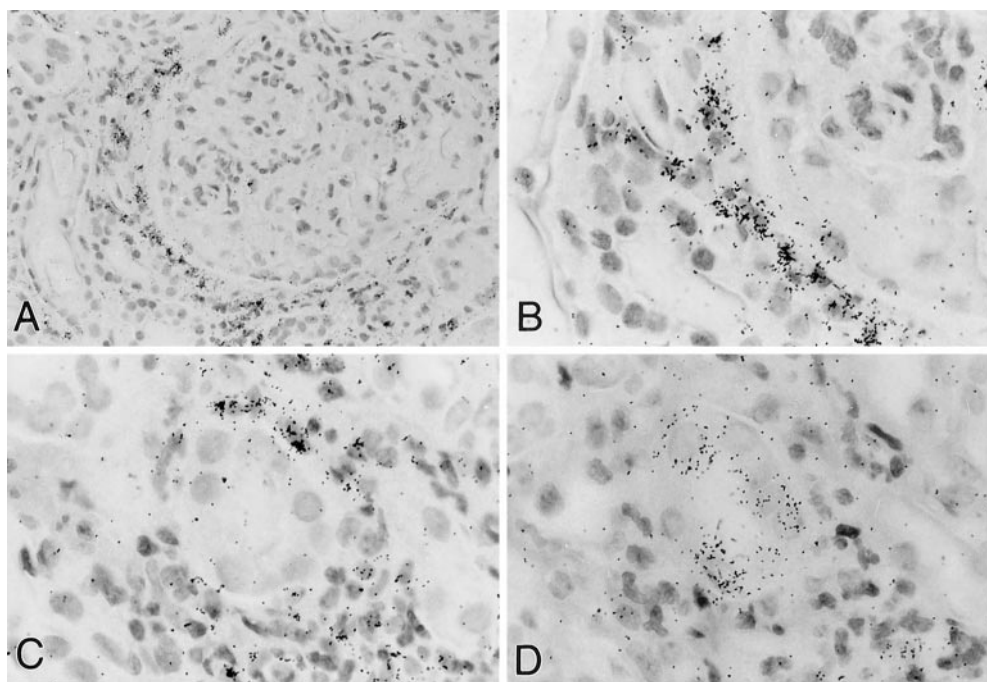


Figure 8. CCR2B mRNA expression in interstitial infiltrating mononuclear cells. (A and B) *In situ* hybridization using a CCR2B antisense riboprobe, demonstrating CCR2B mRNA expression in interstitial infiltrating cells, with prominent clustering around the glomerulus. Original magnification: $\times 400$ in A; $\times 1000$ in B. (C and D) *In situ* hybridization using antisense riboprobes for CCR2B (C) and MCP-1 (D) on replicate sections of a crescentic glomerulonephritis biopsy. Original magnification: $\times 1000$ in C; $\times 1000$ in D. Note the clustering of CCR2 mRNA-positive cells around a tubule, with MCP-1 mRNA expression in the epithelium.

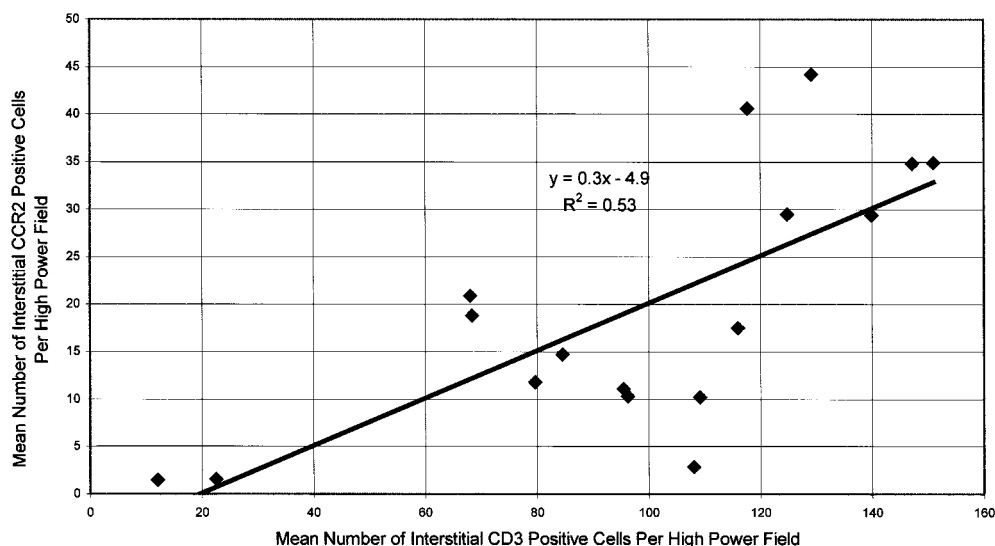


Figure 9. Correlation between the mean number of interstitial CD3-positive T cells and the mean number of interstitial CCR2B mRNA-positive cells ($P < 0.05$).

primary cells (35). The expression of its ligand MCP-1 in renal diseases has recently been reviewed (9). Our findings of MCP-1 mRNA expression by cells in glomerular tufts, crescents, and parietal epithelium, as well as by tubular epithelium and interstitial infiltrating leukocytes, are in accord with previous studies (36,37). Large numbers of glomerular MCP-1-expressing cells were associated with large numbers of glo-

merular CD68-positive macrophages and CCR2B-positive cells, in agreement with the concept of leukocyte chemoattraction by MCP-1. In addition, biopsies with large numbers of MCP-1 mRNA-positive cells contained significantly larger numbers of CD3-positive T cells, at least some of which exhibited evidence of bearing the MCP-1 receptor CCR2B.

In our study, the morphologic distribution of MCP-1 expres-

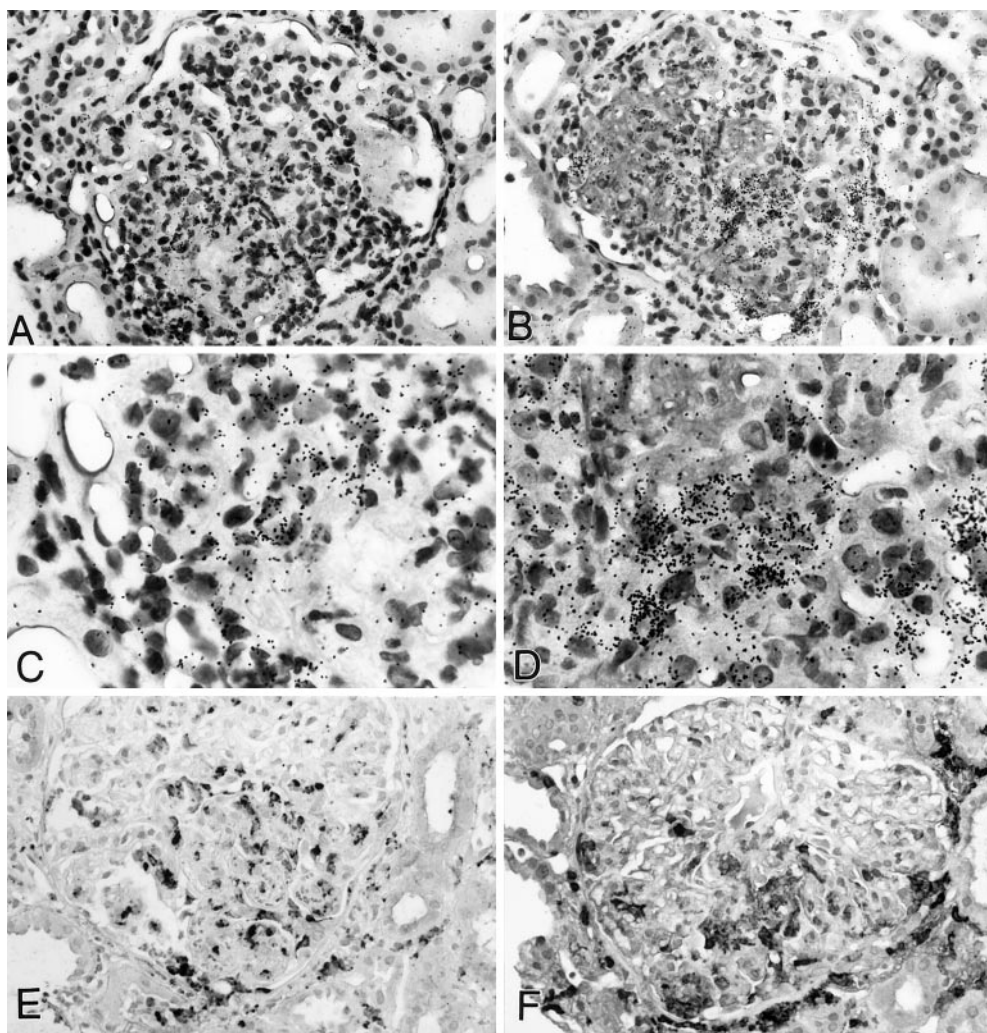


Figure 10. Glomerular MCP-1 and CCR2B mRNA expression. (A and C) *In situ* hybridization using a CCR2B antisense riboprobe, demonstrating CCR2B mRNA expression in infiltrating cells of a severely damaged glomerulus with strong mononuclear cell infiltration. Original magnification: $\times 400$ in A; $\times 1000$ in C. (B and D) *In situ* hybridization using a MCP-1 antisense riboprobe, demonstrating MCP-1 mRNA in a replicate section of the biopsy shown in A. Original magnification: $\times 400$ in B; $\times 1000$ in D. (E) Immunohistochemical analysis of CD68-positive macrophages in a replicate section of the biopsy shown in A, demonstrating prominent glomerular macrophage infiltration. Original magnification, $\times 400$. (F) Immunohistochemical analysis of CCR5-positive cells in a replicate section of the biopsy shown in A. Note the weak diffuse CCR5 signal, with a distribution similar to that of CD68-positive macrophages in E. Original magnification, $\times 400$.

sion, CCR2B mRNA-positive cells, and CD68-positive macrophages is indicative of an inflammatory process in which a subset of glomerular macrophages are attracted via CCR2B. Currently available data on the role of CCR2 and MCP-1 in nephrotoxic serum-induced nephritis, a model of human cGN, are not conclusive. Several studies using MCP-1-neutralizing antibodies have demonstrated beneficial effects on glomerular lesions in rats (38–40) and mice (41). Studies of nephrotoxic serum-induced nephritis in rats demonstrated that an approximately 35% decrease in glomerular macrophages could be achieved by treatment with anti-MCP-1 antibodies (38–40). In contrast to this positive effect, which occurred 4 d after injury initiation, Fujinaka *et al.* (38) observed no differences after 8 d. MCP-1-deficient mice exhibited no differences in glomerular lesions in nephrotoxic serum-induced nephritis, compared with wild-type mice, but did exhibit a significant reduction in tubulointerstitial lesions (42). CCR2-de-

ficient mice demonstrated a reduction in glomerular macrophage infiltrates and proteinuria after 24 h but exhibited a worse disease course after 7 d (43). A recent study of human cGN demonstrated a correlation between the number of crescents and urinary MCP-1 excretion, as well as decreased urinary MCP-1 concentrations after therapy (44).

In a mouse model, blockade of MCP-1 function resulted not only in a reduction of the macrophage infiltration but also in a significant reduction of T cells (41). We observed a significant correlation between the numbers of interstitial T cells and CCR2B-positive cells, as well as significantly larger numbers of T cells, which were localized in areas with more MCP-1-positive cells. Because the ligands for CCR2 and CCR5 attract different subsets of T cells, *i.e.*, MCP-1 preferentially attracts CD4-positive T cells and RANTES preferentially attracts CD8-positive T cells (45), the distribution of CCR2 and CCR5

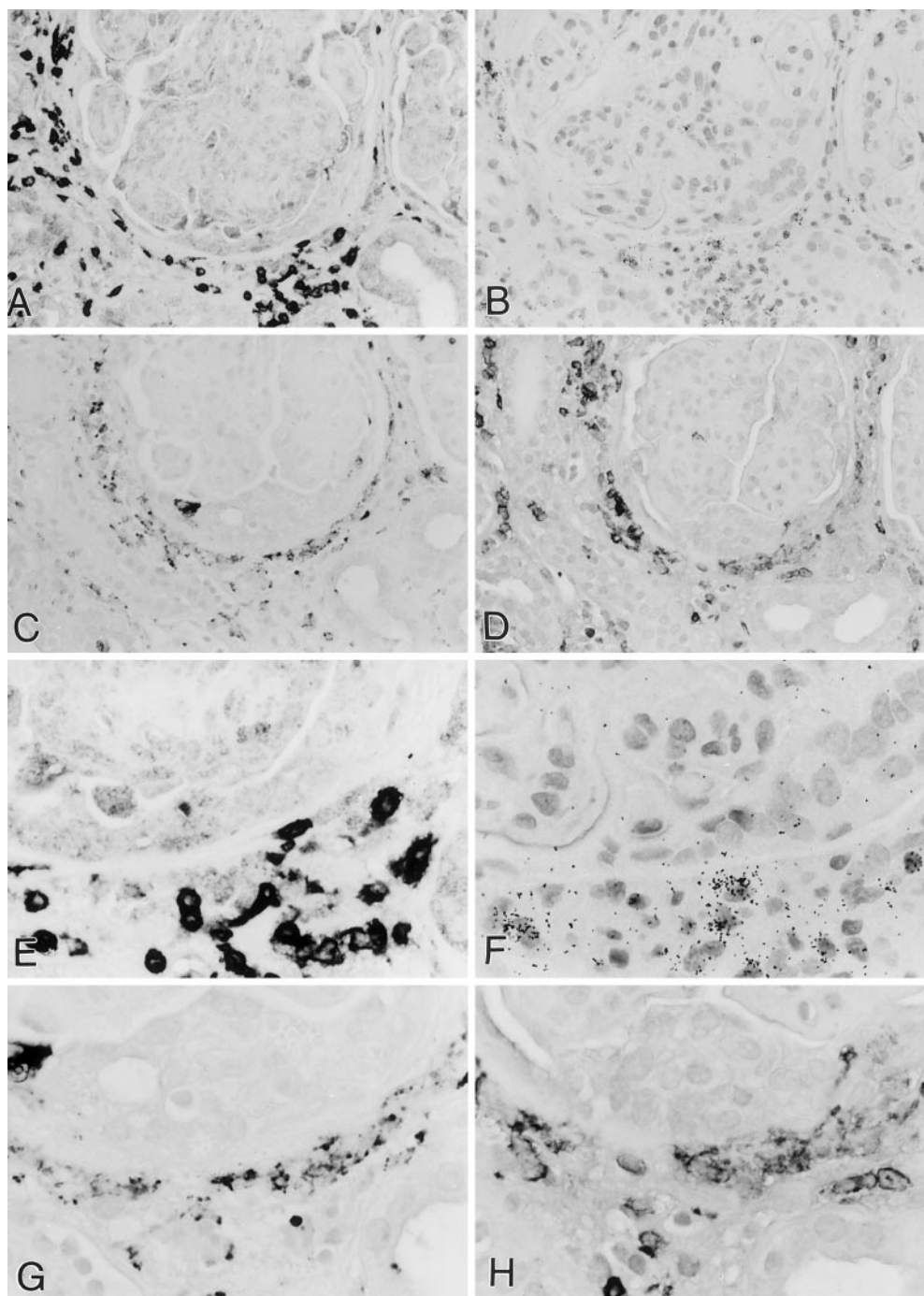


Figure 11. Distribution of interstitial macrophages, T cells, and CCR2B- and CCR5-positive cells. (A and E) Immunohistochemical analysis of CD3-positive T cells, illustrating the distribution of interstitial T cells. Original magnification: $\times 400$ in A; $\times 1000$ in E. (B and F) *In situ* hybridization using a CCR2B antisense riboprobe, demonstrating CCR2B mRNA expression in infiltrating cells in a serial section of the biopsy shown in A. Original magnification: $\times 400$ in B; $\times 1000$ in F. (C and G) Immunohistochemical analysis of CD68-positive macrophages, illustrating a periglomerular layer surrounding a cellular crescent. Original magnification: $\times 400$ in C; $\times 1000$ in G. (D and H) Immunohistochemical analysis of CCR5 in a serial section from the biopsy shown in A. Original magnification: $\times 400$ in D; $\times 1000$ in H. Note that the cell form and distribution of CCR5-positive cells on the left side are better correlated with the T cell distribution (compare A and D), whereas the lower section mirrors the macrophage distribution (compare G and H).

mRNA (and presumably their peptide products) might reflect different subpopulations of CD3-positive cells in the renal interstitium in cGN.

We have demonstrated that CD3-positive T cells contribute to the majority of infiltrating cells in the interstitium but are rare in the injured glomerular tufts in cGN and contribute little to the

cellular composition of crescents. In contrast, macrophages form a major part of the cells in crescents, are often observed to be infiltrating the glomerular tuft, and are also present in large numbers in the interstitium in cGN. These data clearly indicate differences in the infiltrating leukocytic cell populations and in the determinants of these different patterns of anatomic localization. The mismatch between the number of T cells infiltrating glomeruli and the interstitium has been well described (5,13,15,46). In agreement with our data, Hooke *et al.* (13,46) observed no significant increase in intraglomerular T cells in patients with cGN. We observed a subset of crescents that contained three or more T cells. These were usually associated with loss of the capsule separating the glomerulus and the interstitium, suggesting T cell infiltration via the interstitium rather than across glomerular capillary walls. In contrast, the distribution of monocytes/macrophages, which are commonly observed in inflamed glomerular tufts and crescents, indicates a greater tendency of these cells to infiltrate and localize in glomeruli, compared with T cells.

The distribution of subsets of infiltrating leukocytes is reflected in the pattern of CCR2B- and CCR5-positive cells. As described above, CCR2B mRNA-positive cells exhibited a high relative percentage within glomeruli. The patterns of distribution and the overall percentages of infiltrating glomerular leukocytes indicate that monocytes/macrophages are the main CCR2B-positive cell type. In contrast, the relative numbers of CCR5-positive cells mirrored the distribution of CD3-positive T cells, with small numbers in the tuft and large numbers in the interstitium. In a previous study, we demonstrated a similar distribution of T cells and CCR5-positive interstitial cells in various noncrescentic glomerular diseases (15). In addition, a part of the CCR5-positive interstitial cell population matched CD68-positive macrophages with respect to both distribution and numbers. The number of CCR5-positive cells within glomeruli is very small, and the number of glomerular macrophages is far greater than the number of CCR5-positive cells. This suggests either that there is a population of CCR5-negative macrophages that differentially infiltrate glomeruli, rather than the tubulointerstitium, or that CCR5 on glomerular macrophages is downregulated, rendering it undetectable by our methods.

This is the first study of the distribution of CCR2B mRNA-expressing cells in the context of MCP-1 expression, CCR5-positive cells, T cells, and macrophages in human cGN. We propose that the differential expression of chemokines and their receptors mediates, at least in part, different patterns of leukocyte influx into renal parenchymal compartments in acute injury, such as cGN. CCR2B and its ligands seem to be important for glomerular macrophage infiltration, as well as for attraction of a subset of interstitial T cells. In contrast, engagement of CCR5 seems to promote tubulointerstitial infiltration by T cells and a smaller subset of macrophages. These data indicate that therapeutic approaches that block either CCR2 or CCR5 could intervene at different steps in the injury process.

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