RANTES and Monocyte Chemoattractant Protein–1 (MCP-1) Play an Important Role in the Inflammatory Phase of Crescentic Nephritis, but Only MCP-1 Is Involved in Crescent Formation and Interstitial Fibrosis

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Summary

The involvement of chemokines in inflammation is well established, but their functional role in disease progression, and particularly in the development of fibrosis, is not yet understood. To investigate the functional role that the chemokines monocyte chemoattractant protein–1 (MCP-1) and RANTES play in inflammation and the progression to fibrosis during crescentic nephritis we have developed and characterized a murine model for this syndrome. Significant increases in T-lymphocytes and macrophages were observed within glomeruli and interstitium, paralleled by an induction of mRNA expression of MCP-1 and RANTES, early after disease initiation. Blocking the function of MCP-1 or RANTES resulted in significant decreases in proteinuria as well as in numbers of infiltrating leukocytes, indicating that both MCP-1 and RANTES (regulated upon activation in normal T cells expressed and secreted) play an important role in the inflammatory phase of crescentic nephritis. In addition, neutralization of MCP-1 resulted in a dramatic decrease in both glomerular crescent formation and deposition of type I collagen. These results highlight a novel role for MCP-1 in crescent formation and development of interstitial fibrosis, and indicate that in addition to recruiting inflammatory cells this chemokine is critically involved in irreversible tissue damage.

Rapidly progressive glomerulonephritis (GN)[1] is characterized by glomerular inflammation and the formation of glomerular crescents, composed of a pleiomorphic infiltrate of mononuclear inflammatory cells and proliferating parietal epithelial cells. These crescents encroach on the urinary space and compress the glomerular tuft causing acute renal failure. This process is almost always associated with severe interstitial and periglomerular inflammation. Spontaneous recovery is rare. More typically, the inflammatory infiltrate gives way to a progressive fibrotic process involving the crescents and the periglomerular and peritubular interstitium, accompanied by tubular atrophy and progressive renal failure. Indeed, clinical studies of patients with glomerular diseases have shown a close correlation between the degree of interstitial fibrosis and the likelihood of chronic renal failure (1, 2).

Although the pathogenesis of crescentic glomerulonephritis is incompletely understood and likely involves several convergent pathways, there is general agreement that circulating mononuclear phagocytes play a central role (3, 4). Administration of nephrotoxic serum to rats results in a severe proliferative and necrotizing GN that is characterized by glomerular crescent formation and accumulation of leukocytes (5). These infiltrating cells may then release inflammatory mediators that influence the behavior of glomerular, tubular, and interstitial cells. This interaction between infiltrating and resident cells leads to cellular proliferation, matrix expansion, and may ultimately lead to glomerular sclerosis and interstitial fibrosis.

[1]*Abbreviations used in this paper:* CINC, cytokin-induced neutrophil chemoattractant; GN, glomerulonephritis; IP-10, interferon-inducible protein 10 kD; ISH, in situ hybridization; MCP-1, monocyte chemoattractant protein-1; NSS, normal sheep serum; NTS, nephrotoxic serum; RANTES, regulated upon activation in normal T cells expressed and secreted.

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Chemokines are a superfamily of small proteins that are important in recruiting and activating leukocytes during inflammation (6). The chemokines RANTES (regulated upon activation in normal T cells expressed and secreted) and monocyte chemoattractant protein–1 (MCP-1) attract mainly T lymphocytes and mononuclear phagocytes, respectively (7, 8). In vivo, stimulated renal cells are capable of generating both of these chemokines (9, 10, 11), and both have been found to be expressed during models of renal injury (12, 13, 14). Several other chemokines including macrophage inflammatory protein-2 (MIP-2), platelet factor 4, interferon-inducible protein 10 kD (IP-10), and cytokine-induced neutrophil chemoattractant (CINC), have been demonstrated to precede the initial neutrophil influx in experimental anti-GBM GN (15, 16). Studies with neutralizing antibodies show that inhibition of CINC, MIP-2, or MCP-1/JE activity decreases neutrophil accumulation with an associated decline in proteinuria during the first 24 h of disease (17). However, all of these studies concentrate on the acute phase of the disease occurring within the first 24 h of the treatment. Moreover, all of these studies have focused on glomerular changes and do not explore the role that interstitial cells are likely to play in the development of disease throughout the whole cortex. Interstitial cells are important mediators of inflammatory and fibrotic pathways, and the factors that they secrete are likely to be integrally involved in the development of pathology during nephritis (18). Therefore, the in vivo role of chemokines in both disease induction and development remains to be established. In particular, the role of chemokines in the progression from inflammation to fibrosis is unclear.

In this study, we have developed and characterized a murine model of accelerated nephrotoxic serum nephritis, determined the nature of the leukocytic infiltration, and analyzed the expression of chemokines during the progression of acute glomerular injury to glomerular crescent formation and renal fibrosis. In addition, we have determined the functional role of the β chemokines MCP-1 and RANTES in the development of glomerular and interstitial inflammation and crescent formation, as well as in the evolution of the ensuing fibrosis.

Materials and Methods

Preparation of Nephrotoxic Serum. Nephrotoxic serum (NTS) was prepared by immunizing male sheep with a lysate of rat glomeruli, isolated by differential sieving as described previously (19). The sheep serum was heat inactivated at 56°C, absorbed with rat red blood cells and serum proteins, and then filter sterilized. As reported elsewhere (20), NTS has moderate reactivity to type IV collagen and laminin, and substantial reactivity to glomerular cell membrane proteins, particularly β1 integrin and its accompanying α chain.

Induction of Crescentic Nephritis. The protocol for inducing crescentic glomerulonephritis in mice was that of Morley and Wheeler (21), modified for our NTS to avoid rapid lethality from acute renal failure while ensuring adequate tissue injury for analysis. CD1 mice weighing ~30 g were pre-immunized by subcutaneous injection of 200 μg normal sheep IgG in Freund's complete adjuvant. After 5 d, experimental mice were injected intravenously with 50 μl NTS on three consecutive days and controls were injected with 50 μl normal sheep serum (NSS) on the same schedule. At various intervals from 12 h to 3 wk after the first dose of NTS, groups of 6 mice were killed by ether inhalation after an overnight collection of urine in single animal metabolism cages. During this collection, mice were allowed free access to water but not food. At the time of death, serum was obtained by cardiac puncture, and portions of kidney were snap frozen for immunohistology and RNA extraction, or fixed in 4% paraformaldehyde for in situ hybridization or in buffered formalin for routine histology.

Blocking Experiments. Experiments were performed to modulate disease by the administration of mediators that neutralize or modify chemokine function. Thus in separate experiments, groups of mice (n = 6) received daily intravenous doses of either the RANTES antagonist, MetRANTES (22) or hamster anti-MCP-1/JE (23) 30 min before administration of 50 μl of either NTS or NSS. MetRANTES was administered at either 16 or 32 μg per mouse per day, and anti–MCP-1/JE was given at 5 μg per mouse per day. Controls included mice given sterile PBS or 5 μg normal hamster immunoglobulin (Jackson ImmunoResearch Labs., West Grove, PA), respectively. The blocking reagents were given to mice in the stated doses from day 0 to day 6. Mice were then killed on day 7, after 24 h in metabolic cages, and kidneys were removed for morphological and immunohistological analyses, as described below.

Morphological Analysis. Kidney halves were fixed overnight at 4°C in 10% neutral buffered formalin (Fisher), embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin following standard techniques. Sections from NTS mice were examined for glomerular hypercellularity and necrosis, mesangial thickening, formation of glomerular crescents, interstitial infiltrates, and development of glomerular and interstitial fibrosis, as compared to NSS mice. A crescent score was obtained by counting the number of glomeruli showing cellular crescents in at least 100 glomeruli per mouse.

Assessment of 24 h Proteinuria. Urine protein excretion was measured on timed overnight specimens collected at intervals from 3 d to 3 wk from individual mice in metabolism cages and assayed by the sulphosalicylic method (19). Blood urea nitrogen was measured on 200-μl serum samples by the Boston University Medical Center clinical laboratories using an auto-analyzer.

Immunohistochemical Phenotyping and Quantitation of Leukocytes. Kidneys from treated and untreated mice were excised, rolled in Tissue Tek OCT compound (Cryoform, IEC, Needham, MA) snap frozen in liquid nitrogen and stored at −70°C. 4-μm cryosections were cut onto microscope slides, air dried, and then fixed in acetone at 4°C for 20 min. Fixed sections were stained with rat monoclonal antibodies directed against Thy1 (T cells), GR-1 (granulocytes) (both PharMingen, San Diego, CA) and MOMA-2 (mononuclear phagocytes) (Biosource International, Camarillo, CA) using an avidin/biotin staining method. All incubations were carried out under humidified conditions and slides were washed twice between steps for 5 min each in 0.1 M PBS. First, endogenous peroxidase was blocked by incubation for 20 min in methanol containing 0.3% hydrogen peroxide. Nonspecific staining due to crossreaction with endogenous avidin or biotin was then blocked by incubation with avidin solution followed by biotin solution, both for 20 min (Vector, Burlingame, CA). Thereafter, sections were overlaid with 20% normal rabbit serum in PBS for 15 min and then incubated overnight at 4°C with mAbs specific for either Thy1, MOMA-2, or GR-1 (all at 1/10 in 10%

normal mouse serum in PBS) (PharMingen). Bound monoclonal antibody was visualized by incubation with biotinylated rabbit anti–rat immunoglobulin diluted 1/200 in 10% normal mouse serum in PBS, and then streptavidin peroxidase complex (prepared according to manufacturer's instructions) for 30 min each (both Dako, Carpinteria, CA). Finally, slides were flooded with peroxidase substrate solution (400 mg diaminobenzidine in 10 ml of PBS, containing 0.01% hydrogen peroxide) for 10 min. Control slides were included where monoclonal antibody, biotinylated anti-rat immunoglobulin or streptavidin complex were selectively omitted. All slides were counterstained with hematoxylin. For each antibody the number of positively stained cells was determined in 15 high-power fields chosen at random per section (40× magnification; total area 1.8 mm^2) and the average number of cells/field was calculated for each mouse.

Measurement of mRNA Expression by Northern Blots. Total RNA from kidneys obtained from NTS- and NSS-treated mice was isolated using the guanidinium thiocyanate/acid phenol procedure (24). RNA was extracted from PBS-treated mice at the same time points as NTS and NSS mice to determine basal levels of chemokines. Northern blots (25) were prepared with 20 μg of total RNA, purified as indicated above, fractionated in a 1.5% agarose/formaldehyde gel and blotted onto a nylon membrane (Genescreen, DuPont). Membranes were probed using ^{32}P-labeled probes for RANTES (26), MCP-1/JE (27), MIP-1α (28), and TCA-3 (29) applied in 50% formamide hybridization solution at 42°C for 18 h. Blots were washed in 2× SSC/1%SDS at 45°C and exposed at −70°C on Kodak XAR 5 film (Sigma).

Collagen I Deposition. Samples of frozen kidney from NTS- and NSS-treated mice were sectioned at 4 μm, washed in PBS, fixed in acetone, and incubated with polyclonal rabbit anti–mouse type I collagen (Chemicon International, Temecula, CA). The primary antibody was detected with goat anti–rabbit IgG-FITC (Jackson ImmunoResearch Labs.). Both the primary and secondary antibodies were diluted to their optimal working concentration in 2% sheep IgG in PBS. The effectiveness of this blocking procedure was confirmed by a negative staining reaction when a non-specific normal rabbit serum was substituted for the primary antibody. Tissues were also stained for sheep and mouse IgG and mouse C3 using various commercially available FITC-conjugated antisera. Sections were imaged using NIH image 1.56, with 10 randomly chosen fields per section (at a magnification of ×20) being screened to calculate the mean area stained for each section.

Collagen I RNA Expression. These studies used a 600-bp cDNA probe that codes for the COOH terminus and a small part of the untranslated region of the rat α1 type I collagen gene (30) as previously described (31). The cDNA was inserted into and propagated in a pBluescript vector (Stratagene) carrying flanking T3 and T7 RNA polymerase promoters. Once linearized, either of the flanking RNA polymerase promoters could be employed to create radioactively labeled sense or anti-sense riboprobes by a runoff transcription method (25). Runoff transcription products were hydrolyzed in 0.01 M DTT, 0.08 M $NaHCO_3$, 0.12 M $NaCO_3$ for 60 min at 60°C and neutralized in 0.01 M DTT, 0.2 M Na acetate, 0.17 M acetic acid. The product was recovered by salt–ethanol precipitation and Sephadex G-50 chromatography (Pharmacia, Piscataway, NJ). The fractions of interest were pooled, the volume adjusted to give a final specific activity of 3×10^6 cpm/ml in 0.3 M DTT, and stored at −70°C until used. Paraffin sections of 4-μm thickness were cut onto Superfrost®/Plus slides (Fisher Scientific, Pittsburgh, PA), dewaxed, and hydrated. Prehybridization, hybridization, and post-hybridization procedures were carried out exactly as previously described (31). Finally, the sections were coated with Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and sealed in light-tight boxes at 4°C for 7–14 d. The slides were developed with Kodak D-19 (Eastman Kodak Co.), counterstained with Mayer's hematoxylin and mounted using a water soluble mountant. Specificity was evaluated by use of the sense riboprobe or by introduction of an RNAse incubation step during the prehybridization. Sections were examined and photographed with a Nikon Optiphot microscope equipped for dark-field illumination at 100× magnification and bright-field illumination at 100× and 400× magnification (Nikon, Garden City, NY).

Statistical Analysis. The significance of differences between experimental groups was calculated by the Wilcoxon Mann-Whitney test using Xlstat in Microsoft Excel 5.

Results

Morphological Changes in Renal Tissue. The severity of glomerulonephritis was dose dependent and varied from mild glomerular hypercellularity to anuric acute renal failure and early death from extensive glomerular necrosis and crescent formation. There were no differences in histological appearance of kidneys from NSS mice compared with mice given PBS only (Fig. 1 *A*). 12 h after the first injection of NTS, proteinaceous casts were present in renal tubules, coincident with the onset of proteinuria. After 24 h, leukocytes were evident within the interstitium and by day 3 prominent perivascular infiltrates were present (Fig. 1 *B*). At this point the glomeruli were clearly hypercellular with focal areas of necrosis. On day 5 some glomeruli contained crescents and interstitial and perivascular infiltrates were pronounced. On day 7, 55% of glomeruli exhibited prominent cellular crescents (Fig. 1 *C*) and marked abnormalities were present within the tubules and interstitium. These included severely dilated tubules with flattened or denuded epithelia and expansion of the interstitium due to edema, interstitial infiltrates, and the onset of fibrosis (Fig. 1 *D*). On day 14, the fibrotic process was more diffuse throughout the interstitium and had extended to involve some of the glomerular crescents, apparently by invasion through Bowman's capsule.

Analysis of Renal Function. Mice injected with NTS were significantly proteinuric by day 3 and became progressively more so throughout the course of the study (Fig. 2 *A*). Blood urea nitrogen levels rose rapidly during the acute phase of immunologic injury but then improved towards baseline values over the course of 10 d (Fig. 2 *B*). However, concomitant with the development of progressive interstitial and glomerular fibrosis, blood urea nitrogen levels rose progressively from day 14.

Collagen I Expression. Over the course of the disease, both the interstitium and crescents became increasingly fibrotic as demonstrated by Masson trichrome staining (not shown) and deposition of type I collagen (Fig. 3). Most strikingly, a rapid induction of cells expressing mRNA for α1 type I collagen was seen by in situ hybridization (ISH) in perivascular, periglomerular and intervening regions of cortex within 3 d of the final injection of NTS (Fig. 3, *A*

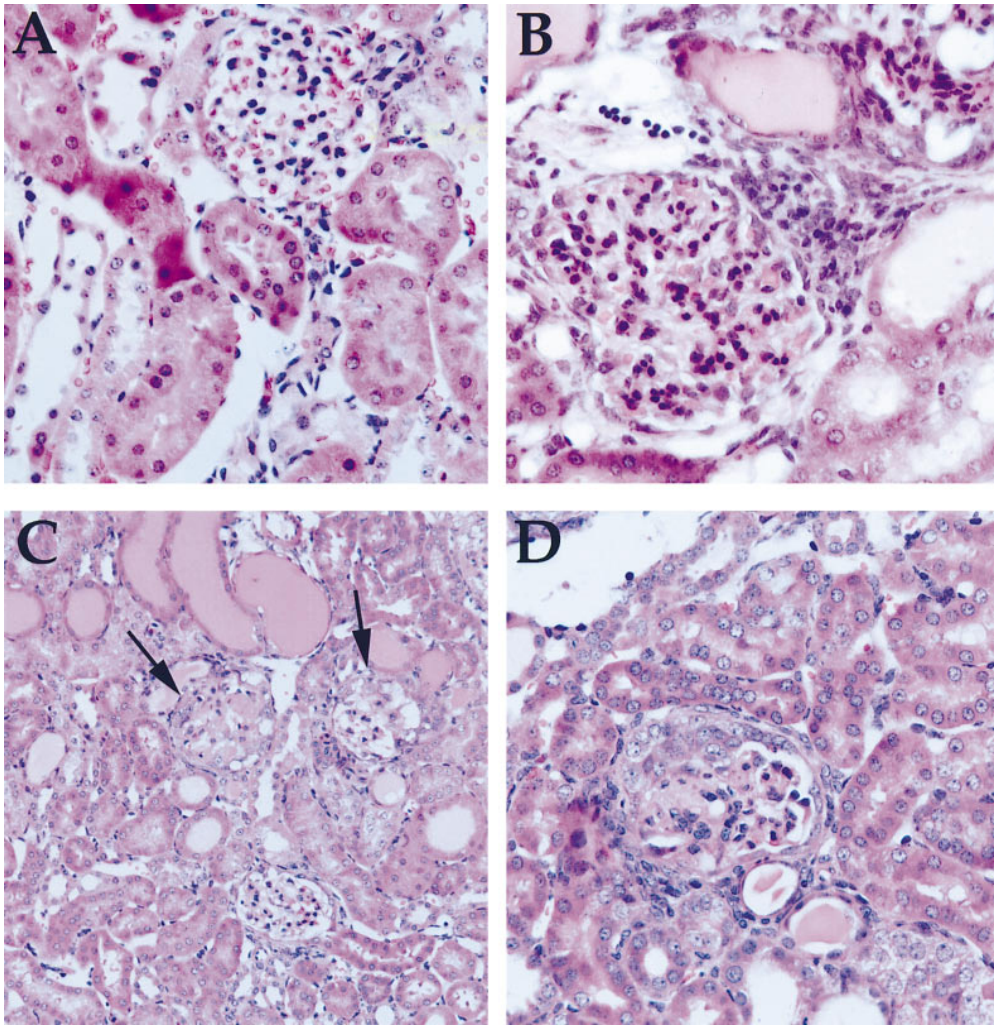

Figure 1. Administration of nephrotoxic serum to pre-immunized mice results in a severe proliferative and necrotizing GN. Hematoxylin and eosin staining of kidney sections from mice given either NSS (*A*) or NTS (*B–D*). NTS mice showed glomerular and interstitial infiltrates (*B*); glomerular crescent formation (*C* and *D*). Arrows highlight crescentic glomeruli. Original magnification: (*A*, *B*, and *D*) ×40; (*C*) ×20.

and *C*). These cells differed from those that constitutively express α1(I) collagen in the perivascular region of normal and control mice and they progressively increased in number, intensity of signal, and extent over the course of disease. Immunofluorescent staining showed deposition of collagen protein in corresponding areas (Fig. 3, *B* and *D*). Initially, glomeruli were negative for type I collagen by IF and ISH, but with time (around day 14), α1(I) mRNA-positive cells encircling the glomeruli appeared to infiltrate the Bowman's capsule of some glomeruli and were followed by positive IF staining of the crescents for type I collagen (data not shown).

Characterization of Inflammatory Infiltrate. Infiltrating lymphocytes, neutrophils and mononuclear phagocytes were identified at different time points during the development of crescentic GN by immunohistochemical staining of frozen sections. (Fig. 4). Increases in both neutrophils and mononuclear phagocytes, but not lymphocytes, were observed 12 h after the first injection of NTS. However, by day 3, all three cell types were increased in kidneys from NTS mice when compared with those from NSS mice at the same time period. Maximal numbers of neutrophils were observed at day 3 of disease, whereas lymphocyte numbers continued to be raised even at day 14. Peak macrophage infiltration was seen at day 7 with a decline in numbers occurring by day 14.

Chemokine Expression. Expression of chemokines involved in the recruitment of leukocytes to sites of inflammation was determined by Northern analysis of RNA isolated throughout the development of crescentic GN (Fig. 5). Therefore, mRNA expression for RANTES ($CD4^+$ T cell and eosinophil chemoattractant) (32); MCP-1/JE (monocyte chemoattractant) (33); MIP-1α (monocyte and eosinophil chemoattractant) (28); and TCA3 (neutrophil and monocyte chemoattractant) (23) was assessed. There was no expression of either MCP-1/JE or RANTES at 12 or 24 h after the induction of disease, but from day 3 onwards NTS treated mice showed strong expression of these chemokines, whereas NSS- or PBS-treated mice did not. There was no detectable expression of TCA-3 in either NTS- or NSS-treated mice (data not shown). Low expression of MIP-1α was detected in all mice, with no discernible difference between mice from NTS or NSS groups.

Blocking Experiments with MetRANTES. MetRANTES was used to neutralize the effect of this chemokine in order to define the functional role that RANTES plays in the devel-

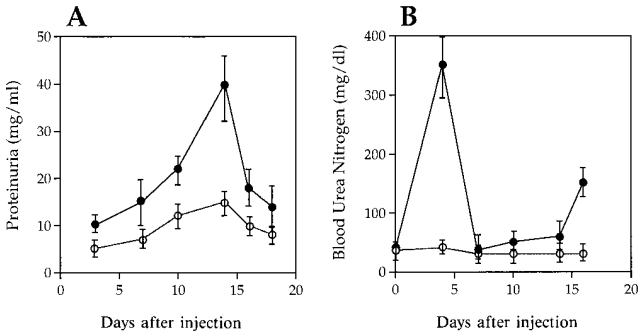

Figure 2. Nephrotoxic serum results in a decline in renal function. Renal dysfunction in mice as shown by (*A*) proteinuria and (*B*) blood urea nitrogen. Each point represents the mean values from either NSS (*open circles*, $n = 6$) or NTS (*closed circles*, $n = 15$) treated mice, and bars show the standard error of the mean.

opment of crescentic GN. Day 7 was chosen to determine the effect of blocking RANTES as by this time there is a significant infiltration by leukocytes, an increase in proteinuria, and the beginning of the development of fibrosis, as marked by collagen I deposition. Thus, 16 μg MetRANTES was administered daily until day 6, when mice were housed in metabolic cages for 24 h before killing on day 7. Four disease parameters were then assessed for the effect of MetRANTES: 24 h proteinuria, leukocyte infiltration, glomerular crescent formation and collagen deposition. First, MetRANTES was seen to induce a 36% decrease in excretion of protein (31 ± 5 mg/24 h in treated versus 52 ± 7 mg/24 h in untreated NTS mice, $P < 0.05$) (Fig. 6 *A*). In conjunction with this there was a 52% decrease in numbers of T cells and a 40% decline in mononuclear phagocyte numbers within sections ($P < 0.005$). However, there was no difference in numbers of neutrophils after MetRANTES treatment (Fig. 6 *B*). A 16% decrease in glomerular crescent formation was counted in H&E stained sections (not significant), although no other changes in morphological abnormalities was seen to occur (Fig. 6 *C*). There was no difference in the deposition of type I collagen after administration of MetRANTES (Fig. 6 *D*). This experiment was repeated with a higher dose of MetRANTES (32 μg/mouse/d) without additional benefit (data not shown).

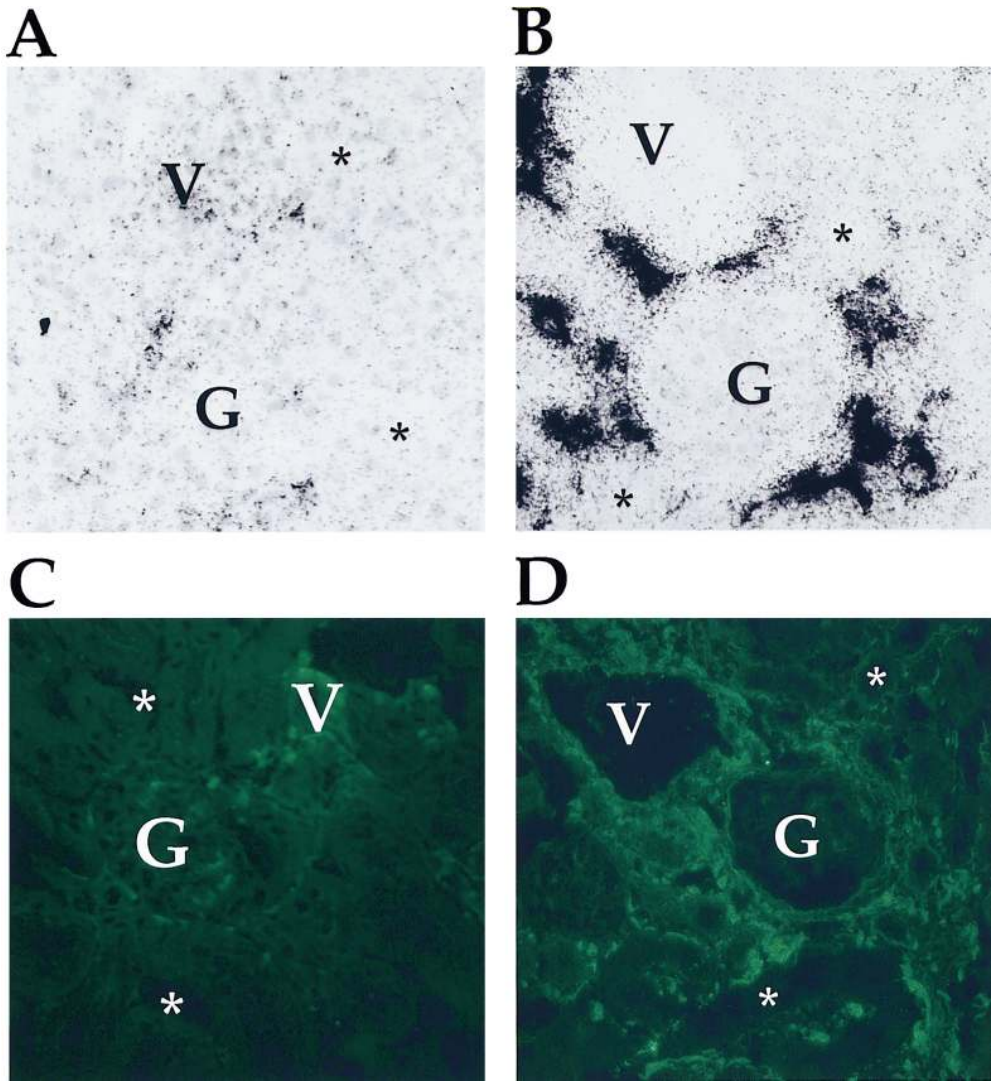

Figure 3. Nephrotoxic serum induces expression of type I collagen mRNA and protein. Deposition of type I collagen in renal tissue isolated from NSS (*A* and *B*) or NTS (*C* and *D*) mice on day 7 of crescentic GN. Plates show (*A* and *C*) a 4-μm paraffin section hybridized with a ^{35}S-cDNA probe encoding a region of the at α1 type I collagen gene and (*B* and *D*) a 4-μm frozen section stained for mouse type I collagen using indirect immunofluorescence. G, glomerulus; *, lumen of tubule; V, venule. Original magnification ×40.

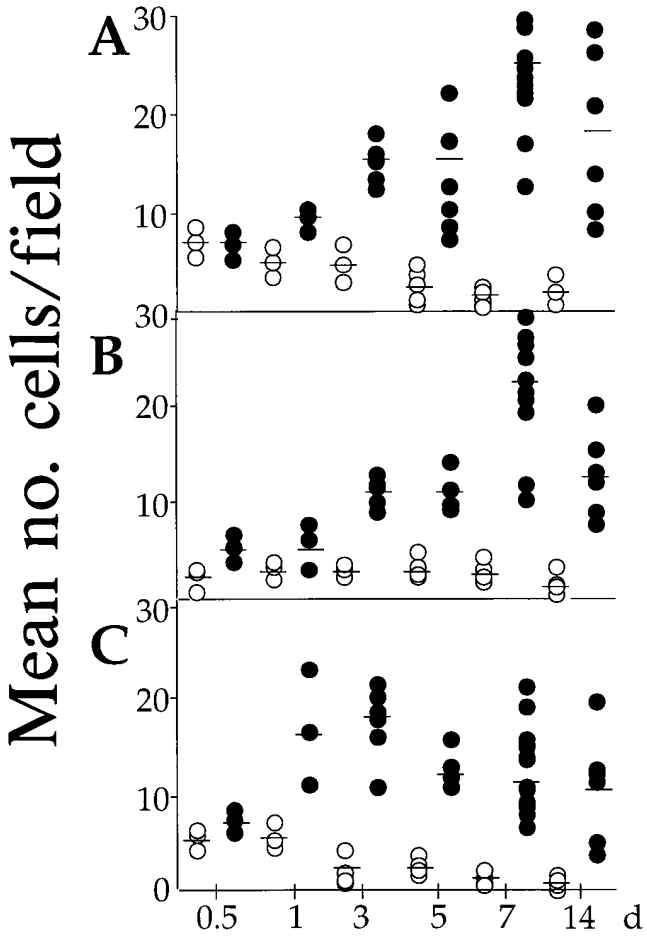

Figure 4. Nephrotoxic serum induces an increase in numbers of leukocytes within renal tissue. Sections were prepared from NSS (*open circles*) or NTS (*closed circles*) mice at various times after disease induction and stained with antibodies specific for T-lymphocytes (*a*), mononuclear phagocytes (*b*), and neutrophils (*c*). Positively stained cells were counted in 15 high power fields per section (total area = 0.5mm^2). Each dot represents one individual mouse analyzed (between 3 and 10 mice per group) with bars depicting means for each group.

Blocking Experiments with Anti–MCP-1/JE. A neutralizing monoclonal antibody specific for MCP-1/JE was used to assess the contribution that this chemokine makes to the development of crescentic GN. Experiments were conducted exactly as described above for MetRANTES, with 5 μg of anti–MCP-1/JE or 5 μg hamster immunoglobulin G (hIgG) being administered daily from day 0 to day 6 to NTS or NSS mice. Hamster Ig had no effect upon the development of disease. Proteinuria was seen to diminish by 25% upon administration of anti–MCP-1/JE ($P < 0.01$) (Fig. 7 *A*). Immunohistochemical phenotyping revealed that neutralizing MCP-1/JE resulted in a 47% decrease in macrophages within both glomerular and the interstitial areas ($P < 0.05$). Similarly, there was a decrease in numbers of lymphocytes ($P < 0.05$). Interestingly, neutralizing MCP-1/JE enhanced the increase in numbers of neutrophils, by 37% compared to untreated NTS mice ($P < 0.01$)(Fig. 7 *B*). Interestingly there was a striking decrease (by 73.5%) in the number of glomerular crescents on day 7 in treated mice compared to untreated NTS mice ($P < 0.001$) (Fig. 7 *C*). Concomitant with this there was a 65% decrease in the deposition of type I collagen in sections from anti–MCP-1–treated NTS mice compared to untreated NTS mice ($P < 0.005$) (Fig. 7 *D*).

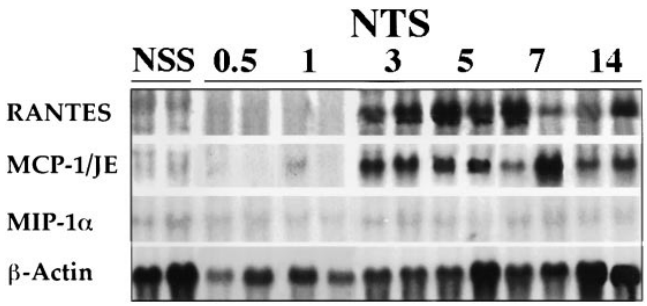

Figure 5. Kidneys from mice with crescentic nepritis show expression of inflammatory chemokines. Northern blot analysis was performed using total RNA (20 μg) extracted from kidneys isolated from NSS or NTS mice throughout disease. Expression of two representative mice at each time point is included. Expression of β-actin was used to determine the quality and quantity of RNA.

Discussion

In this study we have investigated the role of chemokines in the development of glomerular and interstitial inflammation and progression to renal fibrosis in a murine model of crescentic glomerulonephritis. Glomerular and interstitial hypercellularity are the earliest abnormalities in this model of accelerated nephrotoxic serum nephritis (Fig. 1), and they occur concomitantly with the appearance of severe proteinuria and transient acute renal failure (Fig. 2). The inflammatory infiltrate in glomeruli and interstitium is composed of T-lymphocytes, mononuclear phagocytes and neutrophils (Fig. 4), much as has been described in other animal models of crescentic glomerulonephritis (34). Within one week from the onset of disease, glomerular crescents begin to form and there is perivascular and periglomerular type I collagen gene induction (Fig. 3), signifying the onset of renal fibrosis. PMNs, mononuclear phagocytes and lymphocytes may contribute to acute renal cell injury and progression of glomerular disease by a variety of different mechanisms including inducing the secretion of pro-inflammatory mediators such as TNFα and IL-1, which may then induce chemokine expression by resident renal cells. The interaction between infiltrating inflammatory cells and intrinsic renal cells may lead to cell proliferation and matrix expansion which would ultimately result in glomerulosclerosis and interstitial fibrosis.

To determine the role of chemokines in eliciting the migration of inflammatory cells to the kidney in this model, we first examined their expression during the evolution of the lesion and then established the functional role of likely candidates with blocking agents. RANTES and MCP-1 are strongly expressed after disease induction (Fig. 5) at a time

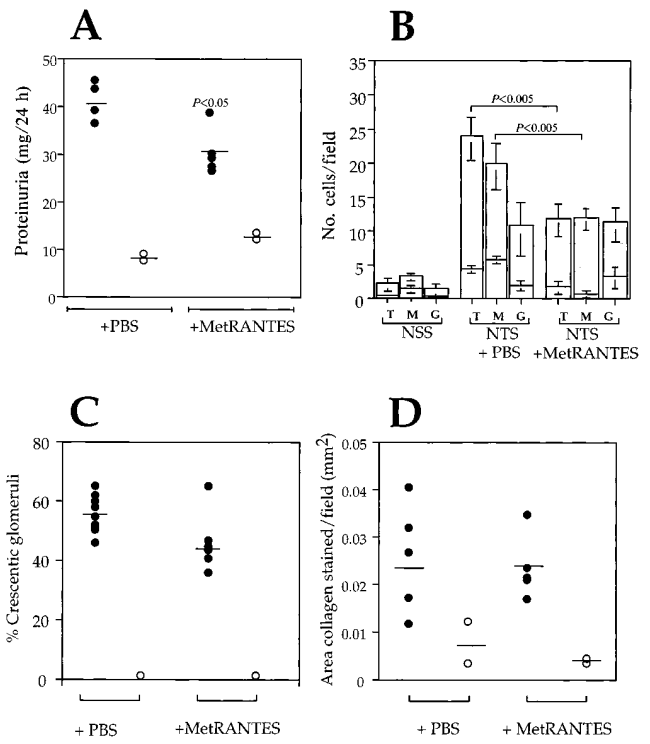

Figure 6. MetRANTES reduces proteinuria and mononuclear cell infiltration. The effects of the RANTES antagonist MetRANTES on development of (*A*) proteinuria, (*B*) leukocyte infiltration, (*C*) glomerular crescent formation, and (*D*) deposition of type I collagen is shown. Groups of NSS (*open circles*, $n = 3$) and NTS (*closed circles*, $n = 6$) mice given 16 μg/day of MetRANTES or PBS were scarified on day 7 of disease. For *A*, *C*, and *D*, each point represents one mouse and bars indicate the mean for each group. For *B*, numbers of T-lymphocytes (*T*), mononuclear phagocytes (*M*) and granulocytes (*G*) were enumerated in immunohistochemically stained sections and bars represent the standard error of the mean for each group. Positively stained cells were counted within the glomeruli (*open bars*) and interstitium (*shaded bars*) of 15 randomly chosen, high power fields within each section. For *C*, one hundred glomeruli were counted in a hematoxylin and eosin-stained section from each mouse, and the percentage of glomeruli containing cellular crescents was calculated. In *D*, kidney sections were stained with an antibody against type I collagen, which was detected with a fluoresceinated secondary antibody. Distribution of immunofluorescence was measured in 10 random fields from each section using NIH image 1.56. For each panel the significance of differences between treated and untreated groups of NTS mice were determined by a Wilcoxon Mann-Whitney test.

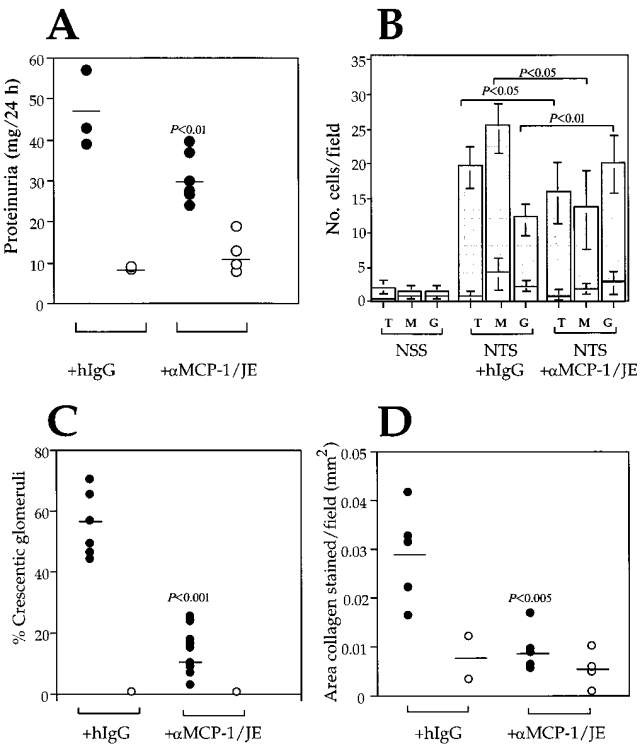

Figure 7. Antibodies to MCP-1 reduce proteinuria and mononuclear cell infiltration, as well as glomerular crescent formation and collagen deposition. The effects of neutralizing antibodies to MCP-1/JE on (*A*) proteinuria, (*B*) leukocyte infiltration, (*C*) glomerular crescent formation, and (*D*) deposition of type I collagen are shown. Groups of NSS (*open circles*, $n = 4$) and NTS (*closed circles*, $n = 10$) were treated daily with 5 μg/d hamster anti–mouse MCP-1/JE or hamster immunoglobulin and were then killed on day 7 of disease. For *A*, *C*, and *D*, each point represents one mouse and bars indicate the mean for each group. For *B*, numbers of T-lymphocytes (*T*), mononuclear phagocytes (*M*), and granulocytes (*G*) were enumerated in immunohistochemically stained sections and bars represent the standard error of the mean for each group. Positively stained cells were counted within the glomeruli (*open bars*) and interstitium (*shaded bars*) of 15 randomly chosen, high power fields within each section. For *C*, 100 glomeruli were counted in a hematoxylin and eosin-stained section from each mouse, and the percentage of glomeruli containing cellular crescents was calculated. In *D*, kidney sections were stained with an antibody against type I collagen that was detected with a fluoresceinated secondary antibody. Distribution of immunofluorescence was measured in 10 random fields from each section using NIH image 1.56. For each panel the significance of differences between treated and untreated groups of NTS mice were determined by a Wilcoxon Mann-Whitney test.

that coincides with the influx of T-lymphocytes and mononuclear phagocytes, and that follows the earlier appearance of neutrophils. MIP1α was found to be constitutively expressed in the kidney and was not upregulated during crescentic nephritis. We did not see any expression of TCA3 during crescentic nephritis, but this may reflect the restricted secretion profile of this particular chemokine (23). MCP-1 and RANTES are both critical mediators in the evolution of inflammatory reactions and are known to be secreted by isolated resident renal cells in vitro (11, 17, 26) as well as by infiltrating inflammatory cells. Expression of both RANTES and MCP-1 has been documented in several human nephritides (35) as well as during experimental anti-GBM GN (14, 17) but these studies are limited to examining glomerular changes occurring during a relatively short time period and do not provide evidence that chemokines are involved in the development of inflammation and disease progression. In our study however, administration of a RANTES antagonist resulted in a decrease in the accumulation of T cells and monocytes/macrophages concomitant with a partial attenuation of proteinuria (Fig. 6). MetRANTES is a functional antagonist of RANTES and blocks calcium mobilization as well as chemotaxis of T cells and monocytes (22). Interestingly, MetRANTES antago-

nizes RANTES and MIP-1α with equal potency in chemotaxis assays but not calcium mobilization. It is thought to act as a competitive inhibitor on the shared MIP-1α/RANTES receptor. We did not find increased expression of MIP-1α in our model of crescentic GN and therefore assume that Met-RANTES is acting primarily to antagonize RANTES, thereby partially blocking the in vivo migration of T cells and mononuclear phagocytes. We have also shown that inhibition of MCP-1 by administration of neutralizing antibodies results in decreased accumulation of mononuclear phagocytes and T-lymphocytes, concomitant with a partial attenuation of proteinuria (Fig. 7). Thus, we have shown that both MCP-1 and RANTES are critically involved in the primary inflammatory phase of crescentic GN.

Progression of disease in mice with crescentic GN was marked by the development of glomerular crescents and the deposition of type I collagen within the interstitium. We have examined the role that MCP-1 and RANTES play in the progression to this later stage of disease. Although administration of a RANTES antagonist resulted in a decrease in the accumulation of T cells and monocytes/macrophages, there was little effect on glomerular crescent formation or deposition of interstitial type I collagen. Previous studies have suggested that activated periglomerular T cells may be involved in the disruption of Bowman's capsule (36), and $CD8^+$ T cell depletion has been found to inhibit crescent formation, (37), but we have shown that RANTES does not appear to be involved in this process. Conversely, neutralizing antibodies to MCP-1 resulted in a striking decrease in both glomerular crescent formation and collagen I deposition. It is interesting that although macrophage accumulation was only inhibited by 50% by antibody administration, this decrease is sufficient to cause such an impressive effect on markers of fibrosis. Since monocyte/macrophage accumulation was not completely inhibited by MCP-1 neutralization, it seems likely that other macrophage-specific chemokines may contribute to this migration. It is not inconceivable that MCP-1 not only affects migration and extravasation of mononuclear phagocytes but mediates direct effects upon the resident renal cells, which in turn affects the pathway to fibrosis. MCP-1 may participate in this fibrotic process directly, by inducing the secretion of extracellular matrix components, or indirectly by instigating the secretion of other pro-fibrotic mediators. It is also possible that MCP-1 alters the phenotype of resident renal cells leading to parietal glomerular epithelial cell proliferation, collagen I production by interstitial fibroblasts, or even tubular epithelial cell transmodulation (38, 39). Whether this is a direct effect of MCP-1, or is mediated by other fibrogenic agents such as TGF-β, PDGF, or FGF, has yet to be determined. We have preliminary data demonstrating the expression of TGF-β mRNA in murine crescentic glomerulonephritis (data not shown), however despite its well-established fibrogenic properties (40), the functional role of this cytokine in post-inflammatory interstitial fibrosis of the kidney has yet to be documented. It is noteworthy that the mice in our model are heavily proteinuric, which some have suggested may be a sufficient stimulus for renal tubular chemokine production and consequent interstitial inflammation and fibrosis (41). On the other hand, proteinuria in the absence of accompanying glomerular inflammation does not appear to stimulate MCP-1 expression (41).

This work has centered on characterizing a murine model of crescentic nephritis and determining the role of the β chemokines MCP-1 and RANTES in the renal inflammation, crescent formation and in the development of the ensuing interstitial fibrosis. We can conclude that both chemokines play a role in the influx of inflammatory cells to the glomeruli and interstitium and in the initial development of renal dysfunction. Moreover, MCP-1 seems to play a critical role in the development of the characteristic glomerular crescents and in the deposition of type I collagen. It is likely that this chemokine mediates direct effects upon intrinsic renal cells which ultimately leads to the formation of crescents and secretion of matrix components.

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