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

By Clare M. Lloyd,* Andrew W. Minto,* Martin E. Dorf,* Amanda Proudfoot,† Timothy N. C. Wells,† David J. Salant,* and Jose-Carlos Gutierrez-Ramos†‡¶

From the *Department of Medicine, Boston University Medical Center, Boston, Massachusetts 02118; †The Center for Blood Research, Incorporated and Department of Genetics and the ‡Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115; §Glaxo Institute for Molecular Biology, Geneva, CH1228 Switzerland; and ¶Millennium Pharmaceuticals Inc., Cambridge Massachusetts 02139

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) is characterized by glomerular inflammation and the formation of glomerular crescents, composed of a pleomorphic 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.

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.

This work was presented in part at the 29th Annual Meeting of the American Society of Nephrology, New Orleans, Louisiana.
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 Nephrotic Serum. Nephrotic serum (NTS) was prepared by immunizing male sheep with a lysate of rat glomeruli, isolated by differential sieving as described previously (19). MetRANTES was administered at either 16 or 32 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 to 3 wk from individual mice in metabolism cages and assayed by the sulfosalicylic acid 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 (Cryoso Inc, E, NC, Needham, MA) snap frozen in liquid nitrogen and stored at −70°C. 4-μm cryosections were cut on 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/biotin solution followed by biotin solution, both for 20 min (Vector, Burlingame, CA). After, 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 mm²) and the average number of cells/field was calculated for each mouse.

Measurement of mRN A Expression by N othern Blots. T otal R N A from kidneys obtained from NTS- and NSS-treated mice was isolated using the guanidinium thiocyanate/acid phenol procedure (24). R N A 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 R N A, purified as described above, fractionated in a 1.5% agarose/formaldehyde gel and blotted onto a nylon membrane (Genescreen, DuPont). Membranes were probed using 32P-labeled probes for RANTE S (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 X AR 5 film (Sigma).

Collagen I Expression. 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.

C ollagen I R N A Expression. These studies used a 600-bp cd N A probe that codes for the C O O H terminus and a small part of the untranslated region of the rat c α1 type I collagen gene (30) as previously described (31). The cd NA was inserted into and propagated in a pBlueScript vector (Stratagene) carrying flanking T3 and T7 R N A polymerase promoters. O nce linearized, either of the flanking R N A polymerase promoters could be employed to create radioactively labeled sense or anti-sense riboprobes by a runoff transcription method (25). R unoff transcription products were hydrolyzed in 0.01 M DTT, 0.08 M NaHCO3, 0.12 M Na CO3, 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 × 106 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 N TB-2 emulsion (Eastman Kodak Co., Rochester, N Y) 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 R N Ase 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, N Y).

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

R esults

M orphological C hanges in R enal T issue. 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.

A nalysis of Renal F unction. M ice 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.

C ollagen I E xpression. O ver 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 mRN A for c 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.
and C). These cells differed from those that constitutively express $\alpha_1$(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), $\alpha_1$(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 raised even at day 14. Peak macrophage infiltration was raised even at day 14. Peak macrophage infiltration was raised even at 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-1$\alpha$ (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-1$\alpha$ was detected in all mice, with no discernible difference between mice from NTS or NSS groups.

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

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 35S-cDNA probe encoding a region of the 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.
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 ng of anti–MCP-1/JE or 5 ng 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). Concomitant with this there was a 47% 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 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 = 0.5 mm²). Each dot represents one individual mouse analyzed (between 3 and 10 mice per group) with bars depicting means for each group.

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 when crescentic glomerulonephritis is fully established in this model. This expression could be the result of an autocrine or paracrine action by infiltrating inflammatory cells. The expression of MCP-1/JE and RANTES in the glomeruli and interstitium is temporally coincident with their detection in renal excretions (35). It is possible that secreted chemokines may have an autocrine or paracrine role in the evolution of crescentic GN.
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).

**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 sacrificed 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.
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 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.

The authors are indebted to Drs. Ramzi Cotran and Jose-Angel Gonzalo for helpful discussion while preparing this manuscript.

This work has been funded by National Institutes of Health grants DK 30932 (D.J. Salant) and HL 148675-02, CICyT PB93-0317, HL94-10-8 and the Aplastic Foundation of America (J.C. Gutierrez-Ramos). J.C. Gutierrez-Ramos is the Amy C. Potter Fellow.

Address correspondence to J.C. Gutierrez-Ramos, Millennium Pharmaceuticals Inc., 640 Memorial Drive, Cambridge, MA 02139. Any queries regarding the murine model of crescentic nephritis should be directed to Dr. D.J. Salant, Renal Section, Department of Medicine, 88 East Newton St., Boston, MA 02118.

Received for publication 18 December 1996.

References

1. Risdon, R.A., J.C. Sloper, and H.E. DeWardener. 1968. Relationship between renal function and histological changes found in renal biopsy specimens from patients with persistent glomerulonephritis. Lancet. 2:363–366.

2. Bohle, A., M. Wehrmann, O. Bogenschultz, W. Vogel, H. Schmitt, C.A. Muller, and G.A. Muller. 1992. The long term prognosis of the primary glomerulonephritides. A morphologic and clinical analysis of 1747 cases. Pathol. Res. Pract.
188:908–924.

3. Thompson, N.M., S.R. Holdsworth, E.F. Glasgow, and R.C. Atkins. 1979. The macrophage in the development of experimental crescentic glomerulonephritis. A m. J. Pathol. 94:223–240.

4. Schreiner, G.F. 1991. The macrophage in glomerular injury. Seminar Nephrol. 11:268–275.

5. Sado, Y., I. Naito, M. Akita, and T. Okigaki. 1986. Strain specific responses of inbred rats on the severity of experimental autoimmune glomerulonephritis. J. Clin. Lab. Immunol. 19:193–199.

6. Schaller, T. J., and K.B. Bacon. 1994. Chemokines, leukocyte trafficking, and inflammation. Curr. Op. Imm. 6:665–873.

7. Schaller, T. J., N. J. Simpson, and J.Y. Mak. 1992. Molecular cloning and expression of the murine RANTES cytokine: structural and functional conservation between mouse and man. Eur. J. Immunol. 22:1477–1481.

8. Rollins, B.J., T. Yoshimura, E.J. Leonard, and J. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete monocyte chemotactic and activating factors (MCP-1, MCP-2, and MCP-3) and their role in the recruitment of monocytes and T lymphocytes. J. Clin. Invest. 86:756–765.

9. Hora, K., J. Sabrano, A. Santiago, T. Morisawa, E. Stanley, Z. Shaheen, and D. Schindler. 1992. Receptors for IgG complexes activate synthesis of monocyte chemotactic and activating factors in human and murine monocyte/macrophages. J. Exp. Med. 176:1621–1638.

10. Sato, M., T. Schuldiner, K. Hori, Y. Xing, Z. Schan, and D. Schindler. 1993. Oxygen radicals induce second messengers for expression of the monocyte chemotactic protein-1. J. Immunol. 150:2253–2259.

11. Wolf, G., S. Alberle, F. Thais, P. Nelsen, A. Krensky, E. Nelson, and R. Stahl. 1993. TNF-α induces expression of the chemotactin cytokine RANTES in cultured mouse mesangial cells. Kidney Int. 44:795–804.

12. Stahl, R., F. Thais, M. Desser, U. Heltmen, and K. Hora. 1993. Increased expression of monocyte chemotactin cytokine protein-1 in anti-thyoxidate antibody-induced glomerulonephritis. Klin. Wschr. 71:1036–1047.

13. Thais, F., U. Heltmen, G. Zahn, H. Haberstroh, N. R. A. Dunkin, and R. Stahl. 1993. Murine RANTES mRNA expression in glomeruli isolated from rats with autologous nephrotoxic serum nephritis. J. Am. Soc. Nephrol. 4:638A (Abstr.).

14. Tang, W.W., S. Yuan, A.J. Wittmer, and M. Qi. 1995. Chemokine gene expression in anti-glomerular basement membrane antibody glomerulonephritis. A m. J. Physiol. 269:F323–F330.

15. Fung, L., Y. Xie, T. Yoshimura, and C.B. Wilson. 1995. Modulation of neutrophil influx in the rat with anti-macrograph inflammatory protein-2 (MIP-2) antibody. J. Clin. Invest. 95:1000–1017.

16. Wu, X., A.J. Wittmer, L.S. Carr, B.A. Cripples, J.E. Delarco, and J.B. Lefkowith. 1994. Cytokine-induced neutrophil chemotactic mediator production and platelet aggregation. J. Clin. Invest. 94:337–344.

17. Tang, W.W., M. Qi, and J.S. Warren. 1996. Monocyte chemotactin cytokine protein-1 mediates granulocye macrophage infiltration in anti-GBM Ab GN. Kidney Int. 50:665–671.

18. Strut, F., and E.G. Nelson. 1994. The role of lymphocytes in the progression of interstitial disease. Kidney Int. 45:S106–S110.

19. Salant, D.J., and A.V. Cybulsky. 1988. Experimental glomerulonephritis. Methods Enzymol. 162:421–461.

20. O’Meara, Y.M., N.atori, A.W. Monti, D.J. Goldstein, E.J. Manting, and D.J. Salant. 1992. Nephrotic syndrome identifies a β1-integrin on glomerular epithelial cells. A m. J. Physiol. 262:F1083–F1091.

21. Morley, A.R., and J. Wheeler. 1985. Cell proliferation within the Bowman's capsule in mice. J. Pathol. 145:315–327.

22. Proudfoot, A., C. Power, A. Hoogewerf, M.O. Montojo, F. Borlat, R. Offord, and T. Wells. 1996. Extension of recombinant mouse RANTES by the retention of the initiating methionine produces a potent antagonist. J Biol Chem. 271:2599–2603.

23. Luo, Y., L. Laning, S. Devi, J. Mak, T.J. Schall, and M.E. Dorf. 1994. Biologic activities of the murine β-chemokine TC-CA3. J. Immunol. 153:4616–4624.

24. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. A nal. Biochem. 162:156–159.

25. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 7:1–7.87.

26. Heeger, P., G. Wolf, C. Meyers, M.J. Sun, S.C. O’Farrell, A.M. Krensky, and E.G. Nelson. 1992. Isolation and characterization of cDNA from renal tubular epithelium encoding murine RANTES. Kidney Int. 41:220–226.

27. Rollins, B.J., E.D. Morrison, and C.D. Stiles. 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor, whose product has cytokine-like properties. Proc Natl. Acad. Sci. USA. 85:3738–3742.

28. Widmer, U., V.D. Yang, S.K.R. Manogue, B. Sherry, and A. Cerami. 1991. Genomic structure of murine macrophage inflammatory protein-1α and conservation of potential regulatory sequences with a human homolog. LD 7812. J. Immunol. 146:4031–4040.

29. Burd, P.R., G.J. Freeman, S.D. Wilson, M. Berman, R. DeKryuff, R.P. Billings, and M.E. Dorf. 1987. Cloning and characterization of a novel T cell activation gene. J. Immunol. 139:3126.

30. Genovese, C., D. Rowe, and B. Kream. 1984. Construction of DNA sequences complementary to rat α- and α2 collagen mRNAs and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. Biochemistry. 23:6210–6216.

31. Minto, A.W., M.A. Fogel, Y. Natori, Y.M. O’Meara, D.R. Abrahamson, B. Smith, and D.J. Salant. 1993. Expression of type I collagen mRNA in glomeruli of rats with progressive hypermellar nephritis. Kidney Int. 43:121–127.

32. Schaller, T., J. Bacon, K.I. Toy, and D.V. Goeddel. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by the cytokine RANTES. N ature (Lond.). 347:669–671.

33. Ernst, C.A., Y.J. Zhan, P.R. Hancok, B.J. Rutledge, C.L. Corless, and B.J. Rollins. 1994. Biochemical and biologic characterization of murine macrophage chemotactic protein-1. J. Immunol. 152:3541–3549.

34. Atkins, R.C., D. J. Nikic-Paterson, Q. Song, and H.Y. Lan. 1996. Modulators of crescentic glomerulonephritis. J. Am. Soc. Nephrol. 7:2271–2278.

35. Lan, H.Y., D. J. Nikic-Paterson, and R.C. Atkins. 1992. Involvement of activated periglomerular leukocytes in the rupture of Bowman's capsule and glomerular crescent pro-
gression in experimental glomerulonephritis. Kidney Int. 67: 743–751.
36. Kawasaki, K., E. Yaoita, T. Yamamoto, and I. Kihara. 1992. Depletion of CD8 positive cells in nephrotoxic serum nephritis of WKY rats. Kidney Int. 41:1517–1526.
37. El Nahas, A.M., E.C. Muchaneta-Kubara, G. Zhang, A. Adam, and D. Goumenos. 1996. Phenotypic modulation of renal cells during experimental and clinical renal scarring. Kidney Int. 49:S23–S27.
38. Okada, H., F. Strutz, T.M. Danoff and E. G. Neilsen. 1996. Possible pathogenesis of renal fibrosis. Kidney Int. 49:S37–S38.
39. Border, W.A., and N.A. Noble. 1994. Transforming growth factor β in tissue fibrosis. N. Eng. J. Med. 331:1286–1292.
40. Eddy, A.A., and J.S. Warren. 1996. Expression and function of monocyte chemoattractant protein-1 in experimental nephrotic syndrome. Clin. Immunol. Immunopathol. 8:140–151.