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Increased severity of glomerulonephritis in C-C chemokine receptor 2 knockout mice

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Increased severity of glomerulonephritis in C-C chemokine receptor 2 knockout mice.

Background. The C-C chemokine receptor 2 (CCR2) is expressed on monocytes and facilitates monocyte migration. CCR2 is a prominent receptor for monocyte chemoattractant protein-1 (MCP-1). This chemokine recruits monocytes to sites of inflammation. It has been suggested that CCR2 and its ligand, MCP-1, play a role in the pathogenesis of glomerulonephritis. The goal of this study was to determine the contribution of CCR2 in a murine model of accelerated nephrotoxic nephritis. We measured the extent of development of renal disease in CCR2 wild-type and knockout mice after the administration of antiglomerular basement membrane antibody.

Methods. Eight groups of animals were treated ($N = 10$ per group). Four days after IgG immunization, CCR2 wild-type and knockout mice received control serum or nephrotoxic serum. The urinary protein/creatinine ratio was measured on days 1 and 3; plasma and kidneys were collected on days 4 and 7. Kidneys were evaluated by light microscopy, immunohistochemistry, and immunofluorescence. The genotype of mice was confirmed by tissue analysis.

Results. Protective effects of CCR2 knockout on the urinary protein/creatinine ratio were observed on day 1, as values for this parameter were significantly lower (35 ± 3.6) than in nephritic wild-type mice (50 ± 6.8). There was a marked increase in proteinuria in nephritic wild-type mice on day 1 compared with vehicle-treated, wild-type animals (5 ± 1.0). On day 3, the ameliorative effects of CCR2 knockout were not observed; the increase in the urinary protein/creatinine ratio was similar in nephritic CCR2 wild-type (92 ± 11.2) and knockout mice (102 ± 9.2). Plasma markers of disease were evaluated on days 4 and 7. At these time points, there were no beneficial effects of CCR2 receptor knockout on plasma levels of urea nitrogen, creatinine, albumin, or cholesterol. On day 7, blood urea nitrogen (248 ± 19.9 mg/dL) and plasma cholesterol were higher in nephritic CCR2 knockout mice than in wild-type mice (142 ± 41.7 mg/dL) that received nephrotoxic serum. Histopathologic injury was more severe in nephritic CCR2 knockout mice than nephritic wild-type mice on day 4 (3.1 ± 0.3 vs. 2.0 ± 0.3) and day 7 (3.6 ± 0.2 vs. 2.9 ± 0.3). By immunohistochemical analysis at day 4, there were significantly fewer mac-2–positive cells, representative of macrophages in the glomeruli of nephritic CCR2 knockout (2.1 ± 0.6) mice than nephritic wild-type (3.9 ± 0.5) animals. By indirect immunofluorescence, there was a moderate, diffuse linear IgG deposition of equivalent severity present in glomeruli of both wild-type and CCR2 knockout nephritic mice.

Conclusion. These results suggest that our strategy was successful in reducing macrophage infiltration, but this model of glomerulonephritis is not solely dependent on the presence of CCR2 for progression of disease. After a transient ameliorative effect on proteinuria, CCR2 knockout led to more severe injury in nephritic mice. This raises the intriguing possibility that a CCR2 gene product ameliorates glomerulonephritis in this murine model. Although effects that occur in chemokine knockout mice are not equivalent to those expected with prolonged use of a chemokine antagonist, this study may nevertheless have implications for consideration of long-term use of chemokine antagonists in renal disease.

Key words: chemokines, monocyte chemoattractant protein-1, nephrotoxic nephritis, inflammation.

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C-C chemokine receptor 2 (CCR2) is expressed by monocytes and is a prominent receptor for monocyte chemoattractant protein-1 (MCP-1) and MCP-3 [1, 2]. CCR2 is expressed in normal mouse kidney and is upregulated in glomerulonephritis (abstract; Schadde et al, *J Am Soc Nephrol* 8:485A, 1997). MCP-1 is a potent agonist for mononuclear leukocytes and is produced by multiple renal cells, including mesangial cells, epithelial cells, and endothelial cells. Renal expression of MCP-1 was reviewed by Luckow, Wolf, and Schlondorff [3]. This chemokine recruits monocytes to sites of inflammation [4, 5]. It has been suggested that CCR2 and its ligand, MCP-1, play a role in the pathogenesis of glomerulonephritis. For example, glomerular expression of MCP-1 mRNA was increased at 3 and 24 hours after induction of Thy-1 nephritis in rats [6]. In antibody-mediated glomerulonephritis, glomerular expression of MCP-1 corre-

lated with the influx of macrophages [7]. In addition, administration of anti–MCP-1 monoclonal antibody significantly suppressed glomerular monocyte/macrophage infiltration and reduced urinary protein excretion in rats treated with nephrotoxic serum [8].

The increased expression of MCP-1 in several disease states has supported the theory that MCP-1 and its primary receptor, CCR2, are responsible for the inflammatory component of these diseases. An association has been demonstrated but not a causal relationship, as the latter requires inhibitors or gene knockout of MCP-1 or its receptor. CCR2 knockout mice have been generated in several laboratories [9–11]. Studies have demonstrated that CCR2 knockout mice failed to recruit macrophages in a peritoneal inflammation model [9] and displayed reduced monocyte migration [10]. We used CCR2 knockout mice in this study to evaluate the contribution of CCR2 to the pathogenesis of accelerated nephrotoxic nephritis.

METHODS

The procedures followed were in accordance with animal-use guidelines of Bristol-Myers Squibb (Princeton, NJ, USA). CCR2 wild-type and knockout mice were generated at Bristol-Myers Squibb [8]. The CCR2 gene was targeted in embryonic stem cells derived from 129/Sv mice. These cells were then aggregated with morula derived from ICR mice in order to generate the CCR2 knockout mice. Thus, the knockout mice have a mixed genetic background of 129/Sv and ICR. The wild-type and knockout mice used in this study were derived from crosses of heterozygous littermates and were of a hybrid 129/Sv:ICR background.

Pups were weaned at 21 days, separated according to gender, weighed, and identified by a transponder placed under the skin. A 0.75 cm piece of tail was obtained and frozen in liquid nitrogen for subsequent analysis of genotype.

At five weeks of age, mice were immunized by an intraperitoneal injection of 0.25 mg sheep IgG (Sigma Chemical Co., St. Louis, MO, USA) in 250 μL phosphate-buffered saline emulsified with an equal volume of complete Freund's adjuvant (GIBCO BRL Products, Gaithersburg, MD, USA). On day 0, four days after IgG immunization, sheep serum (Sigma) or 100 μL of sheep antirat glomerular basement membrane (GBM) nephrotoxic serum (a generous gift from Dr. Karen Munger, Emory University, Atlanta, GA, USA) was administered via a lateral tail vein to wild-type and knockout mice ($N = 10$ per group). Serum was diluted in phosphate-buffered saline so that each mouse received a total volume of 150 μL. Both nephotoxic serum and sheep serum were heat inactivated by heating to 56°C for 45 minutes and kept frozen (−20°C) until needed. Animals were sacrificed on day 4 or day 7.

On days 1 and 3, spot urine samples were collected and analyzed for protein and creatinine. Protein was quantitated using the Coomassie Brilliant Blue system (QuanTtest Blue Total Protein Assay System; Quantimetrix Corp., Redondo Beach, CA, USA) adapted to the Abbott ABA-100 Bichromatic Analyzer (Abbott Diagnostics, Abbott Park, IL, USA). On day 4, one set of mice was weighed and anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneally), and blood was collected from the orbital sinus. The left kidney was removed, cut in half in cross section, and immersion fixed in 10% neutral-buffered formalin for light microscopic examination.

On day 7, the second set of mice was weighed and anesthetized, and tissues and blood were collected as described earlier in this article. Plasma samples were analyzed for albumin, urea nitrogen, creatinine, and total cholesterol using the Boehringer Mannheim/Hitachi 717 Analyzer (Boehringer Mannheim Corp., Indianapolis, IN, USA). Albumin was analyzed using Liquid-STAT Albumin Reagent (Beckman Instruments, Inc., Brea, CA, USA). Blood urea nitrogen (BUN) was analyzed using BUN System Pack (Boehringer Mannheim Corp.). Creatinine was analyzed using Creatinine System Pack (Boehringer Mannheim Corp.). Cholesterol was analyzed using Total Serum Cholesterol (Diagnostic Chemicals Limited, Oxford, CT, USA).

Polymerase chain reaction

The genotype of mice was confirmed by polymerase chain reaction (PCR). Tail tissue from wild-type and knockout mice was digested in PCR buffer containing nonionic detergents with proteinase K for four hours at 55°C with occasional vortexing. Samples were heated to 95°C for 10 minutes and centrifuged for 5 minutes. The supernatant was used as a template and analyzed by PCR.

Polymerase chain reaction was carried out using two pairs of oligonucleotides in a multiplexed reaction. The first pair of oligonucleotides was specific for CCR2, amplifying a 320 bp fragment (5′-TGG TAA ATT CTT CAG GCT TTC C-3′ and 5′-TCC ACA ACC TGA TAA AGC CTC C-3′). The second pair of oligonucleotides amplified a 220 bp fragment of the Neo cassette, used as a positive control (5′-CGG CCA CAG TCG ATG AAT CCA GAA A-3′ and 5′-CCA TTC GAC CAC CAA GCG AAA CAT C-3′). In a total of 30 μL, the reaction mixture contained 20.0 μL milliQ water, 3.0 μL 10 × PCR buffer (Perkin Elmer, Foster City, CA, USA), 1.2 μL dNTPs (2.5 mmol/L), 2.0 μL oligonucleotides, 0.3 μL Perfect Match (Stratagene, La Jolla, CA, USA), 2.5 units *Taq* DNA polymerase (Perkin Elmer), and 3.0 μL template. PCR was carried out using a Mini-

cycler PCR system (MJ Research, Watertown, MA, USA) starting at 94°C for two minutes, then 94°C for one minute, 60°C for 1.5 minutes, and 72°C for one minute for 25 cycles. The resulting reaction products were analyzed by gel electrophoresis (2% agarose) using a 100 bp ladder (GIBCO BRL) as a size marker.

Histopathology, immunohistochemistry, and immunofluorescence

Kidneys were embedded in paraffin, sectioned at 3 μm, stained with periodic acid-Schiff (PAS), and graded by light microscopy without knowledge of the treatment. The glomerular changes were graded as to the severity of injury: grade 0, normal; grade 1, minimal mesangial expansion; grade 2, mild increase in mesangial matrix, segmental in nature and involving less than 50% of the glomerular tuft; grade 3, moderate increase in mesangial matrix that involves greater than 50% of the glomerular tuft; and grade 4, marked increase in mesangial matrix resulting in tuft obliteration.

Paraffin-embedded kidneys were immunohistochemically analyzed using the rat antimouse antibody (1:1000) directed against the macrophage marker mac-2 (Cedarlane, Ontario, Canada). The number of mac-2–positive cells in 20 randomly selected glomeruli from each animal was enumerated and expressed as a mean.

Indirect immunofluorescence was performed on deparaffinized formalin-fixed kidneys after antigen unmasking using 0.1% protease digestion. Biotinylated goat antimouse IgG (1:50; Vector Laboratories, Burlingame, CA, USA) was followed by streptavadin-fluorescein isothiocyanate (1:50; Dako, Carpinteria, CA, USA) for 30 minutes at 25°C. Mice treated with heat-inactivated sheep serum, and duplicate samples from nephritic mice with omission of the primary antibody served as controls.

Statistics

Data are given as mean ± SEM. Renal function data, histopathology scores, and immunohistochemistry data were analyzed by one-way analysis of variance and Tukey's multiple group comparison test. The null hypothesis was rejected at $P < 0.05$.

RESULTS

There was a marked increase in the urinary protein/creatinine ratio in nephritic wild-type mice (50 ± 6.9) on day 1 compared with control wild-type mice (5 ± 1.0). These results are summarized in Figure 1. There was a significant ameliorative effect of CCR2 knockout on this parameter in nephritic animals on day 1 (35 ± 3.6). By day 3, protective effects of receptor knockout were not observed; the increase in the urinary protein/creatinine ratio was similar in the two groups of mice that received nephrotoxic serum.

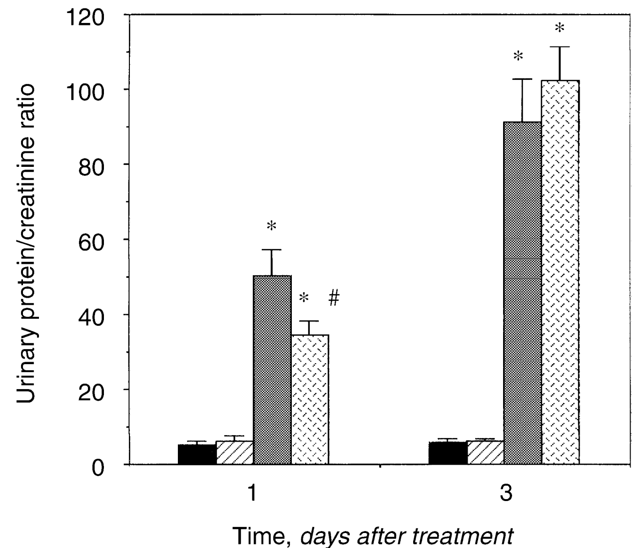

Fig. 1. Effects of CCR2 knockout in nephritic mice on the urinary protein/creatinine ratio. Data are mean ± SEM. $*P < 0.05$ vs. vehicle wild-type (WT); $\#P < 0.05$ vs. nephritic (NTS) WT. Symbols are: (■) vehicle WT; (▨) vehicle knockout (KO); (▩) NTS WT; (▧) NTS KO.

Plasma markers of disease were evaluated on days 4 and 7, and no beneficial effects of CCR2 knockout in nephritic mice were observed. In fact, BUN values were significantly higher in CCR2 knockout mice (111 ± 22.8 mg/dL) on day 4 than in control wild-type mice (25 ± 1.0 mg/dL) and were also higher on day 7 than in nephritic wild-type mice, suggesting more severe injury in the nephritic knockout mice (Fig. 2). Levels of this parameter were not increased in vehicle-treated CCR2 knockout mice.

As with BUN, plasma cholesterol levels were significantly higher in nephritic CCR2 knockout mice (356 ± 25.8 mg/dL) than in nephritic wild-type mice on day 4 (239 ± 37.1 mg/dL) and day 7, as shown in Figure 3. Plasma cholesterol was also increased in wild-type mice given nephrotoxic serum compared with controls.

Plasma albumin decreased significantly in nephritic wild-type and CCR2 knockout mice compared with controls on days 4 and 7, as summarized in Figure 4. This change in plasma albumin is likely due to increased permeability of the GBM and loss of protein in the urine. On day 7, values were significantly lower in nephritic knockout mice than in nephritic wild-type animals.

Plasma creatinine levels were not significantly increased in nephritic wild-type mice on day 4 (0.4 ± 0.02 mg/dL) compared with control wild-type mice (0.4 ± 0.02 mg/dL) or nephritic CCR2 knockout mice (0.4 ± 0.04 mg/dL). On day 7, plasma creatinine levels were slightly increased in nephritic wild-type mice (0.5 ± 0.08 mg/dL) compared with vehicle-treated, wild-type mice

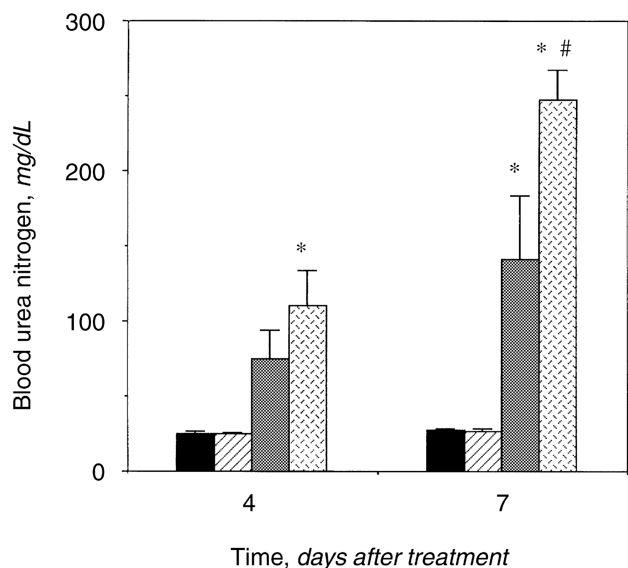

Fig. 2. **Effects of CCR2 knockout in nephritic mice on blood urea nitrogen.** Data are mean ± SEM. $*P < 0.05$ vs. vehicle wild type (WT); $\#P < 0.05$ vs. nephritic (NTS) WT. Symbols are: (■) vehicle WT; (▨) vehicle knockout (KO); (▩) NTS WT; (▦) NTS KO.

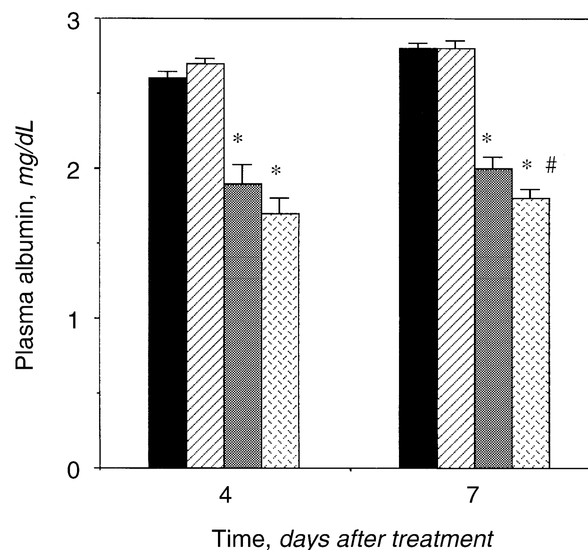

Fig. 4. **Effects of CCR2 knockout in nephritic mice on plasma albumin.** Data are mean ± SEM. $*P < 0.05$ vs. vehicle wild-type (WT); $\#P < 0.05$ vs. nephritic (NTS) WT. Symbols are: (■) vehicle WT; (▨) vehicle knockout (KO); (▩) NTS WT; (▦) NTS KO.

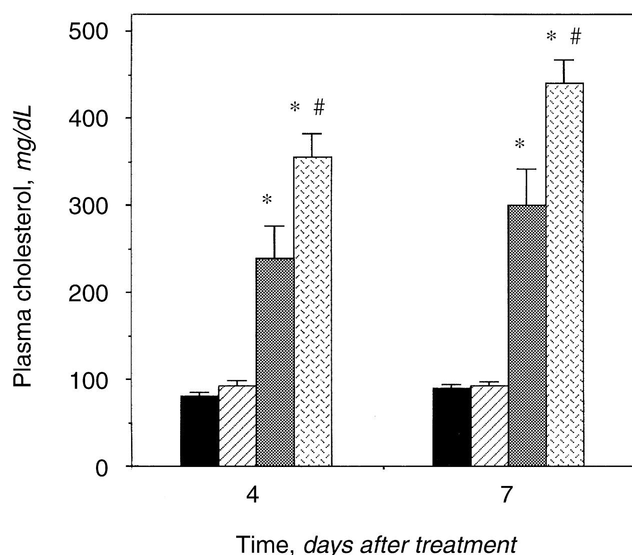

Fig. 3. **Effects of CCR2 knockout in nephritic mice on plasma cholesterol.** Data are mean ± SEM. $*P < 0.05$ vs. vehicle wild-type (WT); $\#P < 0.05$ vs. nephritic (NTS) WT. Symbols are: (■) vehicle WT; (▨) vehicle knockout (KO); (▩) NTS WT; (▦) NTS KO.

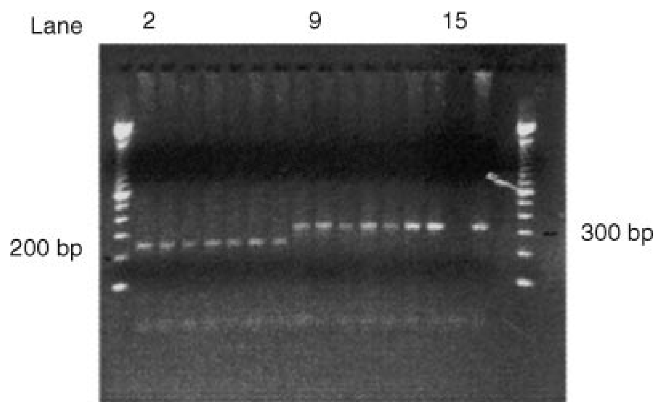

Fig. 5. **PCR was performed using a pair of primers that amplified a 320 bp fragment of CCR2 (lanes 9 through 15, CCR2 wild-type mice) and a pair of primers that amplified a 220 bp fragment of the Neo cassette, included as a positive control (lanes 2 through 8, CCR2 knockout mice).** As expected, the 320 bp bands representing CCR2 are present in samples from CCR2 wild-type mice, but absent in samples from CCR2 knockout mice.

(0.4 ± 0.00 mg/dL), but this change failed to reach statistical significance.

The genotype of CCR2 wild-type and knockout mice was confirmed by PCR analysis. A representative gel is shown in Figure 5. As expected, the 320 bp bands representing CCR2 are present in samples from wild-type mice but are absent in samples from CCR2 knockout mice.

Glomerular injury was significantly more severe in nephritic CCR2 knockout mice on day 4 (3.1 ± 0.3 vs. 2.0 ± 0.3) and day 7 (3.6 ± 0.2 vs. 2.9 ± 0.3) compared with nephritic wild-type mice (Fig. 6). The most prominent glomerular change was an increase in mesangial matrix, which ranged from segmental to global in nature. Additional salient glomerular changes, of slightly greater severity and frequency in nephritic knockout mice, included mesangial cell hypertrophy and hyperplasia, loss of capillary lumina, crescent formation and synechia, and parietal epithelial cell hypertrophy.

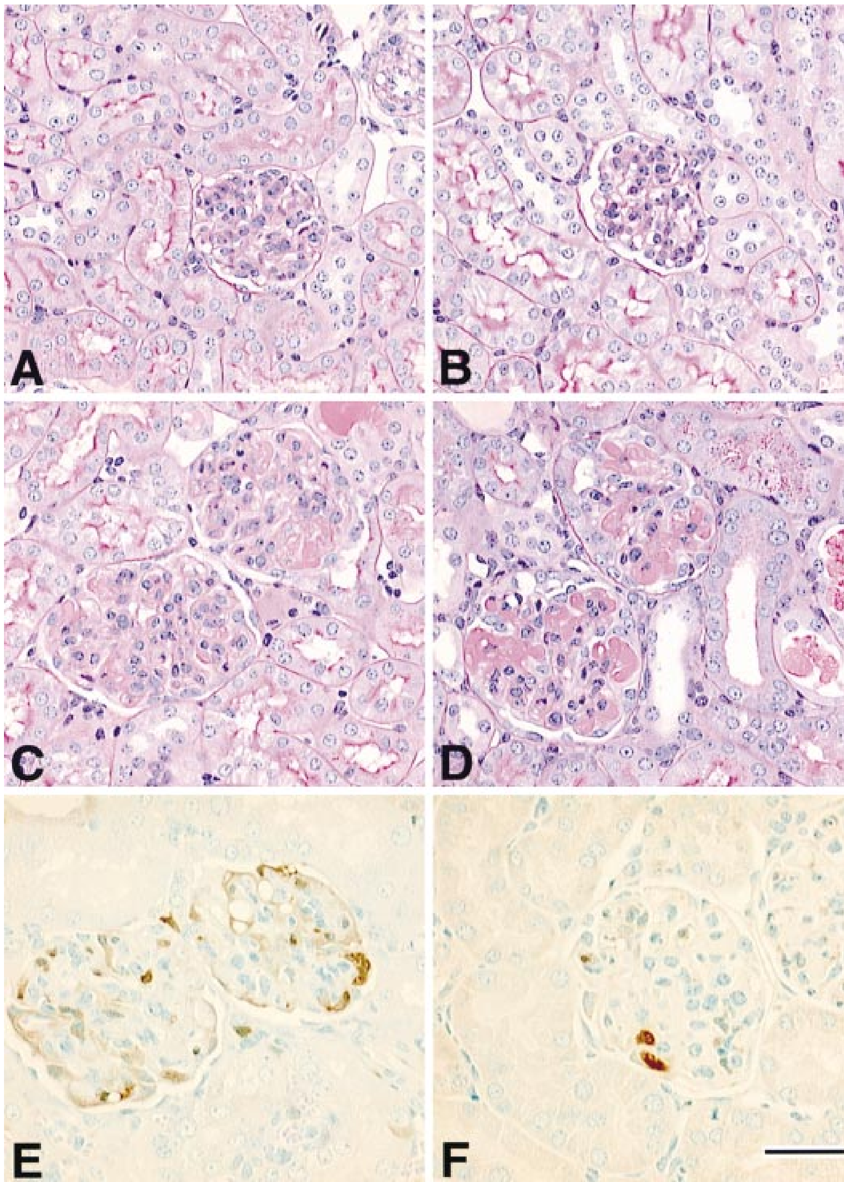

Fig. 6. Glomeruli of vehicle-treated, wild-type (*A*) and CCR2 knockout (*B*) mice were not remarkable on day 4 (periodic acid-Schiff stain). Glomerular injury was less severe in nephritic wild-type mice (*C*) than in nephritic CCR2 knockout mice (*D*). There were more mac-2–positive cells in the glomeruli of nephritic wild-type mice (*E*) than CCR2 knockout nephritic animals (*F*; methyl green counterstain). Bar represents 50 μm.

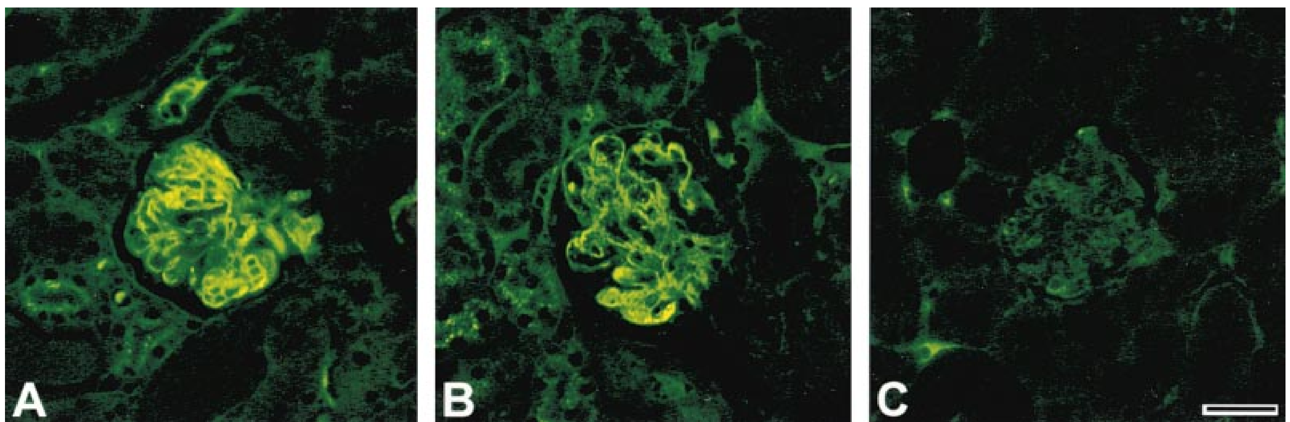

Fig. 7. Generalized, diffuse, linear IgG deposition along glomerular basement membranes, as determined by indirect immunofluorescence, was similar between nephritic wild-type mice (*A*) and CCR2 knockout mice (*B*). Linear immunofluorescence was not present in controls (*C*). Bar represents 100 μm.

The most common extraglomerular change observed in nephritic mice, with a slightly higher frequency in knockout mice as compared with wild type, was dilated tubules containing variable amounts of proteinaceous material, and a rare degenerated neutrophil or sloughed tubular epithelial cells. Prominent resorption droplets were also present in the cytoplasm of proximal tubular epithelial cells. Minimal inflammatory infiltrates occurred multifocally in the interstitium; there was no difference between nephritic wild-type and knockout mice. Interstitial fibrosis was not a prominent feature of this model at this time. The kidneys of CCR2 wild-type and knockout mice treated with heat-inactivated sheep serum were not remarkable.

By immunohistochemical analysis at day 4, there were significantly fewer mac-2–positive cells, representative of macrophages, in the glomeruli of nephritic CCR2 knockout (2.1 ± 0.6) mice than nephritic wild-type (3.9 ± 0.5) animals (Fig. 6). By day 7, there was no difference in the number of mac-2–positive cells within glomeruli of nephritic wild-type and knockout mice.

By indirect immunofluorescence, there was a moderate, diffuse linear IgG deposition of equivalent severity, present in glomeruli of both wild-type and CCR2 knockout nephritic mice (Fig. 7). Immunofluorescence was not observed in mice treated with heat-inactivated sheep serum or in nephritic mice following omission of primary antibody.

DISCUSSION

The increased severity of disease noted in nephritic CCR2 knockout mice in this study was an interesting and unexpected finding, suggesting that a CCR2 gene product may provide an ameliorative effect in nephritis. We originally postulated that the lack of CCR2, the primary receptor for MCP-1, would reduce macrophage number and injury in accelerated nephrotoxic nephritis. The macrophage number was reduced in CCR2 knockout mice as expected, yet increased glomerular injury was observed in nephritic animals. Somewhat similar results were reported by other investigators who found more severe injury in nephritic CCR1 knockout than nephritic wild-type mice (abstract; Topham et al, *J Am Soc Nephrol*, 9:487A, 1998). There are several possible explanations for our finding. First, if chemokines are injected systemically, there is eventual downregulation of the response of target leukocytes [12]. This decrease in target response of leukocytes may be lacking in CCR2 knockout mice, and the absence of downregulation may lead to enhancement of the immune response and greater severity of disease. Because of the complexity of the chemokine family, it is not unlikely that knockout of a chemokine receptor would have significant indirect effects.

Second, other ligands that bind to CCR2, besides MCP-1, may be of benefit in nephritis. MCP-3, MCP-4, and MCP-5 are among the known ligands of CCR2 [13–15], and it seems unlikely that they exert positive effects in nephritis based on described functions [14–16]. For instance, MCP-5 is a potent monocyte active chemokine that is involved in allergic inflammation and the host response to pathogens [15]. However, there may be an as yet unidentified ligand for CCR2 that confers protection in glomerulonephritis.

Third, it has been reported that the severity of glomerulonephritis in mice varies according to the strain. It is important to note that both the wild-type and knockout mice used in this study are of a hybrid 129/Sv:ICR background and are derived from crosses of heterozygous littermates, meaning that they have as close a genetic background as possible. It is extremely unlikely that the differences in renal injury in the wild-type and knockout mice observed in this study are due to differences in their genetic background.

Finally, another consideration is whether the model is different from that previously described. The changes in renal function observed in nephritic wild-type mice in this study are similar to those reported by other investigators. Increased excretion of protein in urine, elevation of plasma cholesterol and BUN, and a decrease in plasma albumin were previously observed in nephritic mice [17]. The structural changes of glomerular expansion and crescent formation observed are also similar to those described previously in rats that received anti-GBM antibody [8].

There was an initial benefit of CCR2 knockout on proteinuria in the very early stages (day 1) of the disease. This correlates with the observations of other investigators that glomerular expression of MCP-1 mRNA peaked between 6 and 24 hours after the induction of anti-GBM disease in rats and that monocyte numbers were maximal at 24 hours [8]. These results suggest that CCR2 and its primary ligand, MCP-1, play an important role in the acute stages of glomerulonephritis.

Our finding of progression of disease in nephritic CCR2 knockout mice from partial protection to lack of effect of receptor knockout is similar to that reported by Fujinaka et al [8]. In that study, the administration of an MCP-1 monoclonal antibody was partially effective in abrogating proteinuria on day 4 after administration of nephrotoxic serum and ineffective at day 8 in Wistar-Kyoto rats. Although the exact time course was different in the two studies, the progression from benefit of anti–MCP-1 treatment to no effect was comparable.

It may seem surprising that the effects of removing the primary receptor for MCP-1 were not more striking, but the role of MCP-1 in generating glomerular damage may be less important than previously considered. There

are several lines of evidence to support this theory. Other C-C chemokines besides MCP-1 may be involved in recruitment and/or activation of leukocytes in nephritis. Glomerular expression of MCP-1, MCP-3, MIP-1α, MIP-1β, and RANTES was observed in nephritic rat glomeruli three days after insult [13]. Similarly, glomerular mRNA of MCP-1, MIP-1α, MIP-1β, and RANTES was increased in renal biopsies from patients with glomulonephritides [18]. When nephritis was induced in MCP-1 knockout mice, no protective effects on glomerular injury were observed, although amelioration of tubular damage was observed [19].

Alternatively, knockout of CCR2 alone might not prevent macrophage infiltration because MCP-1 binds to other receptors such as CCR4. This is borne out by the immunohistochemical analysis indicating that macrophage infiltration was significantly reduced in CCR2 knockout animals, but not totally prevented. Similar partial effects on macrophage infiltration were reported by Fujinaka et al after the administration of anti–MCP-1 antibody [8]. There was a reduction in glomerular macrophage number of approximately 30% in those animals that received anti–MCP-1 antibody.

In addition, both neutrophils and macrophages contribute to the development of glomerulonephritis in this model, so inhibition of monocyte recruitment through CCR2 knockout might not be expected to prevent the disease. In fact, Scandrett, Kissane, and Lefkowith have suggested that acute inflammation in terms of neutrophil influx is more predictive of glomerulosclerosis than glomerular macrophage influx in nephritic rats [20].

Finally, the presence of monocytes alone does not automatically lead to increased injury. Fuentes et al reported that overexpression of MCP-1 in three transgenic mouse lines led to monocyte infiltration, but in all three cases, monocytes were not activated and there was no tissue damage [21].

These results suggest that our strategy was successful in reducing macrophage infiltration, but this model of glomerulonephritis is not solely dependent on the presence of CCR2 for progression of disease. After a transient ameliorative effect on proteinuria, CCR2 knockout led to more severe injury in nephritic mice. This raises the intriguing possibility that a CCR2 gene product ameliorates glomerulonephritis in this murine model. Although effects that occur in chemokine knockout mice are not equivalent to those expected with prolonged use of a chemokine antagonist, this study may nevertheless have implications for consideration of long-term use of chemokine antagonists in renal disease.

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