Monocyte Chemoattractant Protein 1, Macrophage Inflammatory Protein 1α, and RANTES Recruit Macrophages to the Kidney in a Mouse Model of Hemolytic-Uremic Syndrome

Tiffany R. Keepers, Lisa K. Gross, and Tom G. Obrig*
Division of Nephrology, University of Virginia, Box 800133, 1 Lane Road OMS 5815, Charlottesville, Virginia 22903

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The macrophage has previously been implicated in contributing to the renal inflammation associated with hemolytic-uremic syndrome (HUS). However, there is currently no in vivo model detailing the contribution of the renal macrophage to the kidney disease associated with HUS. Therefore, renal macrophage recruitment and inhibition of infiltrating renal macrophages were evaluated in an established HUS mouse model. Macrophage recruitment to the kidney was evident by immunohistochemistry 2 h after administration of purified Stx2 and peaked at 48 h postinjection. Mice administered a combination of Stx2 and lipopolysaccharide (LPS) showed increased macrophage recruitment to the kidney compared to mice treated with Stx2 or LPS alone. Monocyte chemoattractants were induced in the kidney, including monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1α (MIP-1α/CCL3), and RANTES (CCL5), in a pattern that was coincident with macrophage infiltration as indicated by immunohistochemistry, protein, and RNA analyses. MCP-1 was the most abundant chemokine, MIP-1α was the least abundant, and RANTES levels were intermediate. Mice treated with MCP-1, MIP-1α, and RANTES neutralizing antibodies had a significant decrease in Stx2 plus LPS-induced macrophage accumulation in the kidney, indicating that these chemokines are required for macrophage recruitment. Furthermore, mice exposed to these three neutralizing antibodies had decreased fibrin deposition in their kidneys, implying that macrophages contribute to the renal damage associated with HUS.

Shiga toxins produced by enterohemorrhagic Escherichia coli are the causative agents of hemolytic-uremic syndrome (HUS), the primary cause of kidney failure in young children. HUS is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. After colonization of the colonic epithelium, the bacteria secrete Shiga toxins (Stx1 and/or Stx2), which translocate across the basolateral surface of the intestinal epithelium into the bloodstream. The Shiga toxins then travel through the systemic circulation to the kidney, where they cause cellular damage by inhibiting protein synthesis in their target cells (30). The degree of sensitivity of cells to Shiga toxins depends on the relative expression of the Stx-binding globotriaosylceramide (Gb3) receptor on each cell type (21).

Previous reports indicate that Shiga toxins do not inhibit protein synthesis in human monocytes in vitro but rather induce monocytes to secrete the cytokines tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-8 (5, 29, 32). Production of these proinflammatory cytokines by monocytes, particularly TNF-α and IL-1β, has been shown to cause increased expression of the Gb3 receptor on endothelial cells so that more Stx is able to bind, further exacerbating the disease process (13, 14, 31). Further evidence that monocytes are involved in the pathogenesis of HUS has been established by the analysis of HUS patient samples. Several studies have found increased monocyte-produced cytokines, specifically, IL-6, IL-8, and TNF-α, in the sera of HUS patients, indicating that monocytes/macrophages are activated during the disease process. Additionally, detection of IL-6, IL-8, and TNF-α in the urine of HUS patients in higher amounts than in the serum indicates that these cytokines are produced locally in the kidney (9, 10, 33). Evidence of monocyte infiltration into the kidney during HUS was demonstrated by detection of significantly elevated levels of a potent monocyte chemoattractant, monocyte chemoattractant protein 1 (MCP-1), in the urine of HUS patients (33). In addition, biopsy specimens clearly showed the increased presence of macrophages in HUS patient kidneys (33). These data point to the monocyte/macrophage as an important inflammatory mediator in the progression of HUS.

Thus, we have investigated the role of macrophages in a murine model of HUS. We show that macrophages are recruited to the kidneys of Stx2- and/or lipopolysaccharide (LPS)-treated mice in a time-dependent manner and that this recruitment occurs via the release of the chemokines MCP-1 (CCL2), RANTES (CCL5), and macrophage inflammatory protein 1α (MIP-1α) (CCL3) in the kidney. Moreover, neutralization of these chemokines caused decreased renal fibrin deposition, indicating that macrophages, their chemokines, or both are involved in HUS-associated kidney damage.

MATERIALS AND METHODS

Shiga toxin purification. Stx2 was purified by immunoaffinity chromatography from cell lysates (kindly provided by Alison O’Brien) of E. coli DH5α containing the Stx2-producing pES120 plasmid (17). Briefly, Stx2 was purified using 11E10 antibody (26) immobilized using an AminoLink Plus kit (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer’s instructions. Endotoxin was removed using De-toxi-Gel (Pierce Biotechnology) per the manufacturer’s in-

* Corresponding author. Mailing address: Division of Nephrology, University of Virginia, Box 800133, 1 Lane Road OMS 5815, Charlottesville, Virginia 22903. Phone: (434) 982-1063. Fax: (434) 924-5848. E-mail: to3e@virginia.edu.

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structions. Stx2 was negative for endotoxin by a Limulus amoebocyte lysate Pyrotest assay (sensitivity of 0.06 endotoxin units/ml). Stx2 purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Stx2 activity was measured by use of a Vero cell cytotoxicity assay. Although Stx1 and Stx2 have identical enzymatic functions, Stx2 was chosen for these experiments because Stx2 is more frequently associated with clinical isolates of E. coli O157:H7 from HUS patients than Stx1 (20, 22).

**Animal studies.** Male C57BL/6 mice weighing 22 to 24 g were purchased from Charles River Laboratories (Wilmington, MA). Mice were injected intraperitoneally with a low sublethal dose of LPS at 300 μg/kg of body weight (O55:BS; Sigma Chemical Co., St. Louis, MO), 250 ng/kg Stx2 (two times the 50% lethal dose), or a combination of both. These doses were chosen based on previous studies with our mouse model of HUS (11). At 0, 2, 4, 6, 8, 12, 24, 48, and 72 h after injection, two mice per time point were euthanized by CO2 inhalation and kidneys were removed. Kidneys collected at 0 h and kidneys from saline-injected mice were used for controls. Kidneys were processed for RNA, protein, and immunohistochemistry as described below. Time course experiments were repeated at least twice. All animal procedures were done in accordance with University of Virginia Animal Care and Use Committee policies.

**Real-time reverse transcription-PCR (RT-PCR).** One-half mouse kidney was stored in 2 ml RNAlater (Ambion, Austin, TX) at 4°C until RNA extraction. Total RNA was isolated using an RNeasy Midi kit (QIAGEN, Santa Clarita, CA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an iScript cDNA synthesis kit, iQ SYBR green supermix, and a LightCycler (Roche, Basel, Switzerland) according to the manufacturer's instructions. Data were evaluated using Opticon Monitor 3 software (Bio-Rad). Data were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). RT-PCR was performed at least twice. All animal procedures were done in accordance with University of Virginia Animal Care and Use Committee policies.

**Protein analysis.** After removal of mouse kidneys, one-half kidney was placed into 1 ml cold tissue homogenization buffer (50 mM HEPES, 1% Triton X-100, pH 7.4) and protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) was employed. Macrophages were counted in 10 fields of cortex per kidney at ×400 magnification, and an average was calculated for each mouse. Other antibodies used were polyclonal rabbit anti-mouse RANTES (Serotec, Raleigh, NC), polyclonal rabbit anti-mouse MIP-1α (Serotec), and polyclonal goat anti-mouse MCP-1 (R&D Systems). For all immunohistochemistry staining, an avidin-biotin horseradish peroxidase system (Vector Laboratories, Burlingame, CA) was used with diaminobenzidine to give a brown-colored end product at the site of detection. Sections were counterstained in hematoxylin, dehydrated, and mounted. Martius yellow-brilliant crystal scarlet-aneline blue differential staining was performed as previously described (28). Martius yellow and phoshptungstic acid in alcoholic solution stain red blood cells, brilliant crystal scarlet stains muscle and muscle fibrin, and aniline blue stains collagen. Glomeruli positive for fibrin staining were quantified by counting three sets of 20 glomeruli per slide and averaging the percent positive for fibrin at each time point.

**Chemokine neutralization.** Monoclonal rat anti-mouse RANTES (clone 53405), polyclonal goat anti-mouse MIP-1α, and polyclonal goat anti-mouse MCP-1 function neutralizing antibodies and immunoglobulin G (IgG) isotype controls (R&D Systems) were all administered intravenously via tail vein injection at 25 μg per mouse 6 h prior to intraperitoneal administration of 250 μg/kg Stx2 and 300 μg/kg LPS. In the case when more than one antibody was given, mice received 25 μg of each neutralizing antibody mixture per mouse, mice injected with all three neutralizing antibodies received 75 μg of antibodies total. Additionally, rat IgG and goat IgG control antibodies were mixed together at 25 μg and injected into mice for controls where more than one neutralizing antibody was used. Antibody doses were chosen based on previous work by other groups (4). Kidneys were processed for immunohistochemistry as described above. Kidney sections of mice treated with antibodies were evaluated for immune complex deposition according to the method described by McMahon et al., and it was determined that immune complexes were not deposited in the kidneys of these mice (16).

**Statistical analysis.** Data are expressed as means ± standard deviations (SD). Statistical analyses were performed using a two-sample t test. A P value of <0.05 was considered significant.

**RESULTS**

**Macrophages infiltrate the kidneys of Stx2- and/or LPS-injected mice.** C57BL/6 mice were injected with 250 ng/kg Stx2, 300 μg/kg LPS, or a combination of the two agents and sacrificed throughout a 72-h time course. This time course was chosen because disease progression to death in this model is typically 3 to 4 days (11). F4/80 staining of kidney sections showed an increase in macrophages in the kidney after Stx2 and/or LPS injection (Fig. 1). Macrophages were located primarily in the cortex in the intersitial space between the tubules and the capillaries and were also found in the medulla. No evidence of glomerular macrophages was found in any samples. Macrophage accumulation was evident beginning at 2 h after injection and was the greatest at 48 h after injection of either or both agents (Fig. 2). Treatment of mice with both Stx2 and LPS produced greater macrophage influx than either treatment alone. The chronology of macrophage infiltration suggests the induction of one or more chemotactants, first early in the time course, as indicated by the peak in macrophages at 2 to 4 h, and then later in the time course, as indicated by the peaks at 12 and 48 h. Therefore, the temporal induction of monocyte-specific chemokinases was investigated.

**Stx2 and/or LPS induces renal macrophage chemokine mRNA expression.** Investigation of the chemotactic factors involved in Stx2- and/or LPS-induced monocyte recruitment led to the determination that MCP-1, MIP-1α, and RANTES were all elevated in kidneys in the mouse model studied. Chemokine mRNA was determined using quantitative real-time RT-PCR as stated in Materials and Methods. MCP-1 mRNA in the kidney peaked at 2 h after injection of Stx2, LPS, or both (Fig. 3A). LPS and Stx2 plus LPS induced 229-fold and 346-fold increases, respectively, in MCP-1 mRNA at 2 h, which gradually declined until 24 h postinjection. Stx2 induced only a 13-fold increase in MCP-1 mRNA at 2 h compared to control levels, and mRNA was not elevated at any other time point. RANTES mRNA peaked later than MCP-1 mRNA levels (Fig. 3B). Stx2 plus LPS induced a 138-fold increase in RANTES mRNA at 6 h and a 127-fold increase in RANTES mRNA at 8 h subsequent to injection. LPS alone induced a 112-fold increase in RANTES mRNA at 6 h postinjection. Injection of mice with Stx2 alone induced a sixfold increase in RANTES mRNA at 48 h postinjection. The timing of MIP-1α mRNA induction was similar to that of MCP-1 in all treatment groups (Fig. 3C). LPS and Stx2 plus LPS induced a 144-fold increase and a 135-fold increase, respectively, in MIP-1α mRNA at 2 h.
postinjection. Stx2 alone did not cause a significant increase in renal MIP-1α mRNA levels.

Stx2 and/or LPS causes increased renal macrophage chemokine protein expression. Increased renal protein corresponded to the elevated mRNA after Stx2 and/or LPS injection. MCP-1 was highly elevated in kidney tissue homogenate supernatants, as determined by ELISA, with a peak expression at 2 h after Stx2 and/or LPS injection, which slowly diminished to control levels by 72 h (Fig. 4A). RANTES protein peaked at 4 to 12 h after administration of Stx2 and/or LPS (Fig. 4B). MIP-1α renal protein expression was similar to that of MCP-1, as MIP-1α peaked at 2 h after Stx2 and/or LPS administration and returned to saline levels by 48 h postinjection (Fig. 4C). Additionally, MIP-1α, MCP-1, and RANTES renal protein levels were greater in mice injected with the combination of Stx2 plus LPS than in mice injected with either agent alone. Notably, the most abundant chemokine was MCP-1, with a peak renal protein level at 1,121.8 ± 165.6 pg/μg, compared to 503.5 ± 112.4 pg/μg RANTES and 126.6 ± 8.6 pg/μg MIP-1α in mice exposed to Stx2 plus LPS.

Immunohistochemistry confirmed protein expression and
determined the location of the chemokine expression in the kidney. MCP-1 protein was present only in the renal cortex and had a punctate staining pattern in the inner portion of proximal tubules (Fig. 5A and B). RANTES protein exhibited a diffuse staining pattern of the tubules throughout the medulla and cortex (Fig. 5C and D). MIP-1α staining patterns were similar to that of MCP-1, revealing punctate staining of the inner portion of the tubules in both the medulla and the cortex (Fig. 5E and F). It is noteworthy that no staining was found in the glomeruli for any of the chemokines.
Chemokine neutralization in Stx2-plus-LPS-injected mice reduces renal macrophage infiltration and fibrin deposition. In order to determine which chemokines were involved in the induced macrophage infiltration, mice were injected intravenously with 25 μg of each neutralizing antibody to MCP-1, MIP-1α, and/or RANTES 6 h prior to intraperitoneal injection of Stx2 plus LPS. At 48 h after Stx2 plus LPS injection, kidneys were removed and macrophages were counted (Fig. 6A). Administration of anti-MCP-1, anti-MIP-1α, or anti-RANTES antibody alone resulted in a modest reduction (21 to 30%) in

FIG. 5. Chemokines are localized to the tubules of kidneys of Stx2 plus LPS-injected mice. Shown are renal MCP-1 immunohistochemistry of mice injected with (A) saline or (B) Stx2 plus LPS (2 h postinjection for the latter), renal RANTES immunohistochemistry of mice injected with (C) saline or (D) Stx2 plus LPS (6 h postinjection for the latter), and renal MIP-1α immunohistochemistry of mice injected with (E) saline or (F) Stx2 plus LPS (2 h postinjection for the latter). The brown color and the arrows indicate positive chemokine staining. Data are representative of three mice. Experiments were performed in duplicate. All images were taken at ×400 magnification.
infiltrating macrophages compared to levels for IgG controls. Similarly, administration of anti-MIP-1α plus anti-RANTES or anti-MCP-1 plus anti-MIP-1α antibodies resulted in 22.7% and 28.7% reduction in renal macrophages, respectively. However, administration of anti-MCP-1 plus anti-RANTES resulted in a 67.9% ± 17.8% decrease in macrophage infiltration. The anti-MCP-1 plus anti-RANTES and the anti-MCP-1 plus anti-RANTES plus anti-MIP-1α groups both had a statistically significant decrease in renal macrophages compared to levels for the other treatment groups. These data indicate that MCP-1 and RANTES are the major contributors to renal macrophage accumulation in this mouse model. Additionally, mice treated with all three chemokine neutralizing antibodies and injected with only Stx2 had a greater than 80% reduction in infiltrating renal macrophages (data not shown).

In addition to macrophage staining, kidney sections from these chemokine-neutralized mice were also stained for fibrin deposition. Increased fibrin deposition is an indication of kidney damage and decreased renal filtration. After sections were stained using the Martius yellow-brilliant crystal scarlet-aniline blue differential stain as described in Materials and Methods, mice were evaluated according to the glomeruli positive for fibrin as indicated by bright red staining. There was a significant reduction in fibrin deposition in the glomeruli and in the cortex when all three chemokine-neutralizing antibodies were administered prior to Stx2 plus LPS injection of the mice (Fig. 6B). The reduction in fibrin deposition was evident not only in the glomeruli but also in the cortex and medulla of mice administered all three chemokine-neutralizing antibodies (Fig. 6C and D). This decrease in fibrin deposition as well as the decrease in macrophage infiltration suggests that macrophages are involved, at least in part, in causing the kidney damage associated with HUS.

**DISCUSSION**

We have previously established a mouse model of HUS that exhibits the triad of hemolytic anemia, thrombocytopenia, and renal failure that defines the disease (11). In the present report, we use this mouse model to investigate the role of the macrophage during the development of HUS. We describe here the progression of macrophage infiltration into the kidney...
These data are a strong indication that MCP-1 and RANTES are neutralizing RANTES, MCP-1, and MIP-1 (20 to 30%) in Stx2 plus LPS-induced renal macrophage accumulation. Furthermore, we show that the combination of Stx2 plus LPS induces a greater influx of macrophages and higher chemokine expression in the kidney than either Stx2 alone or LPS alone. These data indicate that these two agents produce an additive effect when administered together.

After determining that macrophages were recruited to the kidneys of mice injected with Stx2 and/or LPS in a time-dependent manner, we sought to determine the chemokines responsible for this infiltration. Previous studies have shown that mice or rats injected with LPS have increased macrophage infiltration and increased levels of renal MCP-1 and RANTES (6, 36). Additionally, mouse models of sepsis, a disease in which macrophages are involved, have shown increased levels of MCP-1, RANTES, and MIP-1α in organs including the kidney, liver, and lungs (15, 18). Analysis of our model revealed that these three chemokines were upregulated in the kidneys after Stx2 and/or LPS injection. It is noteworthy that while administration of Stx2 alone did not greatly alter renal mRNA levels of any chemokine, it did cause significantly increased protein expression. It is likely that posttranslational control or storage of the chemokines in renal cells allows for rapid secretion of these chemokines upon exposure to Stx2 without upregulation of gene transcription (3, 23, 24). These data suggest that Stx2 and LPS induce renal cells to produce MCP-1 and MIP-1α, resulting in the initial macrophage infiltration, and to produce RANTES later in the time course, resulting in the continued macrophage infiltration of the kidney.

Immunohistochemistry for these chemokines revealed that all three chemokines were located in tubular cells and that none were in the glomeruli. These data correlated with the fact that macrophages were located only in the tubular interstitium and did not infiltrate the glomeruli. It is not surprising that these chemokines are produced by tubular epithelial cells, considering previous studies documenting the ability of these cells to produce various cytokines and chemokines, including MCP-1, RANTES, and MIP-1α (1, 37). Additionally, renal tubular cells have been shown to be a primary target of Stx in animal models and in cell culture, inducing both apoptosis and cytokine secretion (8, 12, 19, 34, 35).

MCP-1 and RANTES were the most abundant renal chemokines induced by Stx2 plus LPS. Thus, it was hypothesized that MCP-1 and RANTES were the primary inducers of the Stx2 plus LPS-induced renal macrophage infiltration. To test this hypothesis, mice were treated with neutralizing antibodies to MCP-1, RANTES, and MIP-1α in all possible combinations prior to Stx2 plus LPS injection. A previous study by Fillon et al. demonstrated that neutralizing antibodies to MCP-1, RANTES, and MIP-1α reduced macrophages in the lungs of pneumococcus-infected mice by 33% only when administered together, not separately (4). In contrast, we found that administration of these chemokines separately resulted in a modest reduction (20 to 30%) in Stx2 plus LPS-induced renal macrophage accumulation. Furthermore, neutralizing both RANTES and MCP-1 proved to be as effective at significantly reducing renal macrophages as neutralizing RANTES, MCP-1, and MIP-1α. These data are a strong indication that MCP-1 and RANTES are the primary chemoattractants involved in Stx2 plus LPS-induced renal macrophage infiltration. A complete inhibition of macrophage infiltration was not observed; however, it is likely that, at the doses used, the chemokines are not completely neutralized by the antibodies. Alternatively, other chemokines may be involved in the macrophage infiltration seen in our model. Of note, gene array analysis done by our lab has shown that the RNA of the macrophage chemokine IP-10/CXCL10 is upregulated in mouse kidneys (see the supplemental material in reference 11). The role of this chemokine in our HUS mouse model is currently under investigation.

To determine whether macrophage inhibition had any effect on kidney function, kidneys from mice administered anti-MCP-1, anti-RANTES, and anti-MIP-1α antibodies prior to Stx2 plus LPS injection were evaluated for fibrin deposition. Fibrin-rich thrombi in the renal microvasculature are associated with HUS and diminished kidney function in these patients (2, 27, 30). Furthermore, it has been shown, by using a model of experimental glomerulonephritis in the rabbit, that macrophages can induce glomerular fibrin deposition (7). We have also shown previously in our HUS mouse model that an increase in renal fibrin deposition is associated with an increase in serum creatinine and a decrease in kidney function (11). Using a differential stain for fibrin, we found that mice administered all three chemokine-neutralizing antibodies and Stx2 plus LPS had significantly decreased glomerular and total kidney fibrin deposition. This likely indicates that the recruited macrophages or the chemokines RANTES, MIP-1α, and MCP-1 contribute to diminished renal function and to the pathology of the HUS disease state. In support of this conclusion, Palermo et al. found that deleting mice of splenic and liver macrophages reduced Stx2 plus LPS-induced mortality (25).

In summary, we have demonstrated that macrophages infiltrate the kidneys of an HUS mouse model primarily via the chemokines MCP-1 and RANTES. Currently, we are investigating whether it is the macrophages or the chemokines that contribute to the increased fibrin deposition found in our model. Additionally, we are evaluating whether depletion of macrophages or monocytes from the circulation will have any effect on renal disease and survival. Although we have not specifically investigated the usefulness of chemokine neutralization as a therapy for HUS in a more clinical setting, experiments that would test the efficacy of such treatment after Stx2 and LPS exposure are a priority for our laboratory. Nonetheless, the data presented here suggest that neutralizing these macrophage chemokines may be a useful therapeutic for ameliorating the kidney damage associated with HUS.

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REFERENCES


