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Role of Macrophages and Colony-Stimulating Factor-1 in Murine Antiglomerular Basement Membrane Glomerulonephritis

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ABSTRACT

Macrophages have been shown to mediate glomerular injury in antiglomerular basement membrane (anti-GBM) glomerulonephritis in rats and rabbits. To evaluate the role of macrophages and the macrophage-related cytokines, colony stimulating factor-1 (CSF-1), monocyte chemoattractant protein-1 (MCP-1) and RANTES, accelerated anti-GBM nephritis was studied in \textit{op/op} mutant mice, which lack CSF-1 and are severely macrophage deficient, and in heterozygous \textit{op/+} control mice. Observations were made 24 h and 3 days after the injection of rabbit anti-mouse GBM antibody in mice preimmunized with rabbit immunoglobulin G. Proteinuria rose progressively in both groups but did not differ between them (urine protein/creatinine ratio at 3 days: 1.01 ± 0.38 in \textit{op/op} versus 1.45 ± 0.43 in \textit{op/+}; \textit{P}, not significant). In both \textit{op/op} and \textit{op/+} mice, anti-GBM nephritis was associated with renal expression of mRNA for RANTES and MCP-1 and barely detectable levels of mRNA for CSF-1. In contrast, these cytokines were not expressed in sham-injected mice. Morphologic lesions appeared earlier in \textit{op/op} mice but were comparable by Day 3. Glomerular injury consisted of capillary thrombosis and endothelial cell damage associated with mild to moderate leukocyte infiltration. Despite enhanced expression of mRNA for RANTES and MCP-1, glomerular macrophage infiltration was not increased in \textit{op/+} mice. It was concluded that, in mice, in contrast to rats and rabbits, accelerated anti-GBM nephritis may develop in the absence of both CSF-1 and macrophage infiltration.

Key Words: Antiglomerular basement membrane nephritis, experimental glomerulonephritis, macrophages, colony stimulating factor-1

Macrophages play a key role in mediating glomerular injury in antiglomerular basement membrane (anti-GBM) glomerulonephritis in the rabbit and rat. The complement-independent autologous phase of anti-GBM nephritis in these species is characterized by extensive glomerular macrophage accumulation resulting from an influx of circulating monocytes (1-13). Glomerular macrophages are locally activated to elaborate chemotactic peptides, cytokines, proteolytic enzymes, and other inflammatory mediators (1,7,9,14). When glomerular macrophage infiltration is prevented by monocyte depletion, proteinuria and morphologic injury are ameliorated (2,4-6,8,9).

The chemotactic factors responsible for complement-independent glomerular macrophage influx and activation in anti-GBM nephritis are unknown. Several monocyte-specific cytokines synthesized by mesangial cells are potential candidates, including colony stimulating factor (CSF-1), monocyte chemoattractant protein (MCP-1), and RANTES (regulated upon activation, normal T cell expressed and secreted) (15,16).

In view of the critical role of macrophages in anti-GBM nephritis, it was of interest to study this disease in the \textit{op/op} mouse, a mutant strain that lacks CSF-1 because of a mutation on chromosome 3 within the gene coding for CSF-1 (17-19). The absence of CSF-1 results in severe deficiencies of bone marrow macrophages, blood monocytes, tissue macrophages, and osteoclasts (17-19). We postulated that the \textit{op/op} mouse would be an ideal model in which to study the roles of CSF-1 and of macrophages in mediating glomerular injury in anti-GBM nephritis.

METHODS

Production of Anti-Mouse GBM Antibodies

GBM was prepared from Swiss mouse kidneys (Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, the Netherlands) by a differential sieving technique, followed...
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by sonication and detergent treatment as previously described (20). Antisera against this basement membrane suspension were raised in rabbits. The pooled antisera were heated at 56°C for 45 min, and the immunoglobulin G (IgG) fractions were purified by affinity chromatography on a Sepharose 4B-coupled-protein-A column (Pharmacia, Upsala, Sweden). The IgG antibodies were concentrated by ultrafiltration with an XM-50 Dialow membrane (Amicon Corp., Lexington, MA), sterilized by passage through a sterile 0.2-μm-pore-size filter, and stored at -30°C. The IgG concentration was measured by the radial immunodiffusion technique.

Induction of Anti-GBM Nephritis

Anti-GBM nephritis was induced in 12- to 16-wk old male op/+ mice (N = 10) and op/op mice (N = 10). Animals were obtained from an established colony (17,19). Seven days before the administration of anti-GBM antibody, the mice were preimmunized with 0.2 mg of normal rabbit IgG administered ip with complete Freund’s adjuvant (1:1 in a total volume of 0.2 mL). Nephritis was then induced by the iv injection of rabbit anti-mouse GBM antibody (0.2 mg in a total volume of 0.3 mL). Four nephritic mice in each group were euthanized after 24 h, and six additional nephritic mice in each group were euthanized at 3 days. Other op/+ and op/op mice (N = 2 in each group) were preimmunized in an identical manner, sham injected with 0.3 mL of NaCl, and euthanized 3 days later. Additional preimmunized op/+ and op/op mice were sham injected with normal rabbit IgG (0.2 mg in a total volume of 0.3 mL) (N = 4 in each group). Proteinuria did not differ between sham-injected mice given saline versus normal rabbit IgG, and these data were combined.

Clinical Parameters

Beginning 7 days before the administration of saline or anti-GBM antibody and continuing until the day of euthanasia, daily urine specimens were obtained from mice for the measurement of urinary protein excretion. Urinary creatinine and white blood cell count.

Immunohistology

Kidney fragments were snap frozen in liquid nitrogen, and 2- to 4-μm cryostat sections were incubated with monospecific, fluorescein-labeled goat anti-mouse Ig (heavy and light chains) and goat anti-mouse C3 serum (both from Cappel Laboratories, West Chester, PA), goat anti-rabbit Ig (Nordic, Tilburg, The Netherlands) absorbed with 500 mg/mL of lyophilized nonimmune mouse serum, and rabbit anti-human fibrinogen cross-reacting with mouse fibrinogen (Dako, Copenhagen, Denmark). The sections were examined in a fluorescence microscope equipped with epilumination, (Leitz, Wetzlar, Germany), and the staining intensity was recorded semiquantitatively (grading 0 to +4) as previously described (20).

Electron Microscopy

For electron microscopy, small pieces of cortex were fixed in 2.5% glutaraldehyde (Sigma), dissolved in 0.1 M sodium cacodylate (Sigma) buffer, pH 7.2, for 4 h at 4°C, and washed in the same buffer. The tissue fragments were postfixed in phosphate-buffered 2% OsO₄ (Johnson Matthey Chem., Roystone, United Kingdom) for 2 h, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were cut in an ultratome (LKP Produkter, Bromma, Sweden) and stained with 5% uranyl acetate for 45 min and with lead citrate for 2 min at room temperature. The sections were examined in an electron microscope (ELMISKOP 101; Siemens, Berlin, Germany).

Northern Blot Analysis

mRNA levels of RANTES, CSF-1, and MCP-1 were evaluated on Days 1 and 3 in control and nephritic animals. Total RNA was extracted from the left kidney, and samples of 10 μg were then used for northern blot analysis. After electrophoresis on a 1% agarose gel, the RNA was transferred to nylon filters. Labeling of the cDNA probes specific for CSF-1, MCP-1, RANTES, GAPDH, and 18S ribosomal RNA was accomplished by random-prime transcription with [32P]dCTP (16,25,26). Nylon filters were prehybridized (65°C, 1 to 3 h) in a solution containing 1 M NaCl and 1% sodium dodecyl sulfate and were then hybridized with the labeled cDNA probes for 16 h. After the filters were washed to high-stringency (0.1× SSPE [150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA; pH 7.4] 1% sodium dodecyl sulfate), autoradiography with intensifying screens (~70°C) was performed. Hybridization of the specific mRNA was quantitated by laser densitometry, and results were normalized to the respective GAPDH bands. All studies were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Statistical Analysis

Data are expressed as the mean ± SE. Values were compared with the t test for paired or unpaired data or analysis of
RESULTS

Clinical Parameters

Body weight was lower in op/op mice than in op/+ mice at the time of entry into the study (32 ± 2 versus 23 ± 4 g; P < 0.01). In sham-injected op/op mice (N = 6), the baseline Ur/UCr × 10, calculated as the average of daily values obtained on 7 days before sham injection, was 0.15 ± 0.04. Ur/UCr averaged 0.12 ± 0.03 over the 3 days after the injection (P, not significant [NS] versus baseline) (Figure 1). In sham-injected op/+ mice (N = 6), Ur/UCr was 0.21 ± 0.05 during the week before sham injection and 0.23 ± 0.06 after (P, NS versus baseline). At each time before and after sham injection, proteinuria was greater in op/+ mice than in op/op mice (P < 0.05).

Baseline Ur/UCr, calculated as the average of daily values obtained on 7 days before the administration of rabbit anti-GBM antibody, was again higher in op/op than in op/op (0.17 ± 0.03 in op/+ versus 0.09 ± 0.02 in op/op; P < 0.05) (Figure 1). Ur/UCr did not differ between the two groups when measured 24 and 48 h after the administration of anti-GBM antibody (Figure 1). At the time of euthanasia on Day 3, Ur/UCr was 1.01 ± 0.38 in op/op versus 1.45 ± 0.43 in op/+; P, NS. In both nephritic groups, proteinuria was significantly greater than baseline values (P < 0.01 at 48 and 72 h, paired t test) and also significantly greater than values in the corresponding sham-injected groups (P < 0.01 at 48 and 78 h).

Serum creatinine did not differ among the groups at the time of euthanasia (data not shown). Serum anti-rabbit antibody titer, measured 24 h after the administration of rabbit anti-mouse GBM antibody, was 90 ± 8 in op/op versus 95 ± 7 in op/+ (P, NS). When measured 3 days after the administration of anti-GBM antibody, serum anti-rabbit antibody titer was 93 ± 6 in op/op versus 215 ± 55 in op/+ (P < 0.05). Serum anti-rabbit antibody titers in sham injected op/op and sham-injected op/+ mice were undetectable on Days 1 and 3.

mRNA

Several immune-mediated glomerular diseases have been associated with changes in the expression of leukocyte-activating cytokines such as CSF-1, MCP-1, and RANTES (27–32). We therefore performed northern blot analysis on RNA extracted from whole kidneys harvested from our experimental animals. As shown in Figure 2, sham-injected op/op (N = 2) and op/+ (N = 2) contained no detectable mRNA for CSF-1, MCP-1, or RANTES, but had comparable levels of mRNA for the constitutionally expressed GAPDH. In contrast, kidneys from anti-GBM-injected op/op and op/+ mice expressed detectable levels of mRNA for RANTES and MCP-1 at 24 and 72 h after the induction of glomerulonephritis. Levels for CSF-1 were barely detectable. No consistent differences in the expression of RANTES or MCP-1 were detectable by inspection or densitometry of the northern blots between op/op and op/+ mice after 24 h (four mice examined per group) or after 72 h (six mice examined in each group). In fact, mRNA levels for RANTES tended to be higher in op/op mouse kidneys. Thus, anti-GBM nephritis in both op/op and op/+ mice is associated with the renal expression of mRNA for RANTES and MCP-1. In contrast, neither of these cytokines, nor CSF-1, is expressed in control, sham-injected mice.

Morphology

Light Microscopy and Electron Microscopy. In the op/op group, glomerular lesions were absent on Day 1, whereas in the op/+ group, minor to moderate damage could already be observed. These lesions consisted of segmental deposition of homogeneous or finely granular periodic acid-Schiff-positive material in the capillary loops, intravascular coagulation, endothelial cell damage, and necrosis. At Day 3, glomerular damage was more extensive and identical in both groups (Figure 3). At this time, occasional epi-
Figure 2. Steady-state mRNA for CSF-1, RANTES, MCP-1, and GAPDH in total RNA extracted from the whole kidneys of sham-injected and anti-GBM antibody (Ab)-injected op/op and op/+ mice. Comparable results were obtained in four experiments for Day 1, six experiments for Day 3, and two sham experiments. Kb, kilobase.

The glomerular neutrophil infiltration in op/op mice was 0.5 ± 0.3 PMN/glomerular cross-section on Day 1 and increased to 1.7 ± 0.9 PMN/glomerular cross-section on Day 3. Although neutrophil infiltration in op/+ mice was greater on Day 1 than on Day 3, the intragroup variation was very large (1.9 ± 3 versus 0.7 ± 0.4 PMN/glomerular cross-section). Glomerular PMN accumulated predominantly in necrotic areas. Glomerular neutrophilic infiltration in sham-injected op/op and op/+ mice was low and comparable (0.2 ± 0.0 versus 0.2 ± 0.1 PMN/glomerular cross-section, respectively). The glomerular lesions, especially the deposition of fibrin and endothelial cell damage, were confirmed at the electron microscopic level (Figure 4).

Immunohistology. All kidneys showed strong linear staining of the GBM for rabbit and mouse IgG (3+) (Figure 5A and B). Fibrin deposits varied but paralleled roughly the segmental necrotic lesions seen by light microscopy (Figure 5D). C3 deposits were also variable (Figure 5C). In addition to minor mesangial deposits in all glomeruli, which is a normal finding in mice, more extensive C3 deposits could be observed along the glomerular capillary wall in a fine granular pattern and in the mesangial areas of several kidneys. Sham-injected mice of both strains showed only occasional minor C3 and Ig deposits in the mesangium.

The op/op mouse carries a mutation on chromosome 3 within the gene coding for CSF-1 (17–19). As a result, the serum of op/op mice lacks CSF-1 (18). Normally, CSF-1 regulates the survival and proliferation of mononuclear phagocytes and is responsible for normal macrophage and osteoclast maturation (17–

DISCUSSION

There is considerable evidence that glomerular injury in the autologous phase of anti-GBM nephritis in the rat and rabbit is mediated by infiltrating glomerular macrophages (1–13). In accelerated models of anti-GBM nephritis, macrophage infiltration of the glomerulus begins within 24 h after antibody administration and peaks within 2 to 3 days (3,6,8,11,12). The macrophage dependence of autologous-phase glomerular injury in rat and rabbit models has been demonstrated by experimental maneuvers that induce monocytopenia (2,4–6,8,9). These maneuvers reduce glomerular macrophage infiltration and ameliorate or prevent proteinuria and histologic abnormalities in the rat and rabbit (2,4–6,8,9).

The op/op mouse carries a muta...
The absence of CSF-1 results in severe deficiencies of bone marrow macrophages, blood monocytes, tissue macrophages, and osteoclasts (17–19). Kidney tissue macrophages in op/op mice are reduced to 3% of the numbers present in op/+ mice at 2 months of age and to 28% of the numbers present in op/+ mice at 3 months of age (19). We postulated that the op/op mouse would be an ideal model in which to study the roles of CSF-1 and of macrophages in mediating glomerular injury in anti-GBM nephritis.

Glomerular injury in our model of accelerated autologous-phase anti-GBM nephritis occurred in op/op mice, despite the absence of CSF-1 and the absence of glomerular macrophage infiltration. Moreover, glomerular injury in control op/+ mice also occurred in the absence of glomerular macrophage accumulation. These observations suggest that glomerular injury in the early autologous phase of murine anti-GBM nephritis does not require CSF-1 or macrophage infiltration. However, we cannot exclude the possibility that
the few macrophages present in the glomerulus, activated by the up-regulated glomerular chemokines, may have played a role in mediating renal injury.

Mouse anti-rabbit antibody titer was significantly higher at Day 3 in op/+ mice injected with rabbit anti-rat GBM antibody than in similarly treated op/op mice. The finding of similar intensity of immunofluorescence staining for mouse Ig in the glomeruli of op/+ and op/op mice, despite increased serum anti-rabbit antibodies in op/+ mice, may be explained if glomerular binding sites for anti-rabbit antibody were saturated in both strains of mice during the autologous phase. Impaired antibody response in op/op mice is not surprising, in view of the important role macrophages play in antigen processing and presentation. Although the increased antibody response in op/+ mice was associated with earlier morphologic injury, by Day 3, the severity of glomerular injury was comparable in both groups of mice.

Monocyte-specific cytokines synthesized by mesangial cells, such as CSF-1, MCP-1, and RANTES, have been shown to play a role in mediating glomerular injury in several experimental models of renal disease, including anti-GBM nephritis (28–32). However, in our studies, glomerular injury developed in CSF-1-deficient op/op mice. Moreover, macrophage infiltration did not increase in nephritic op/+ mice, despite enhanced renal expression of mRNA for the potent monocyte chemoattractants MCP-1 and RANTES. Because we did not localize the source of the increased mRNA to glomeruli versus tubules, our observations do not necessarily reflect a dissociation between monocyte chemoattractant expression and biologic response. In addition, it is possible that an enhanced message for these cytokines was not translated into increased protein product.

We conclude that, in contrast to the mechanisms operative in the rat and rabbit, glomerular injury in the autologous phase of anti-GBM nephritis can occur
in the mouse in the absence of glomerular macrophage infiltration and in the absence of CSF-1.

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