Abstract. Endotoxemia leads to the infiltration of inflammatory cells in glomeruli and the tubulointerstitium of the kidney. The ultimate mechanisms for this infiltration, however, are not entirely clear. In this study, the glomerular formation of the chemokine RANTES (regulated upon activation normal T cell expressed and secreted) was examined in an in vivo model of endotoxemia to evaluate the role of the local release of chemokines in the regulation of this inflammatory cell infiltration. Since the beneficial effects of nitric oxide (NO) on immune-mediated tissue injury have been reported, we also examined possible interactions between the chemokine release and the L-arginine/NO pathway. Lipopolysaccharide (LPS) was injected intraperitoneally, and RANTES was assessed by Northern blotting, a chemotactic assay, and a specific enzyme-linked immunosorbent assay.

Endotoxin-induced acute inflammation is characterized by increased vasopermeability, platelet aggregation, emigration of inflammatory cells, and the formation of microthrombosis. Responsible mediators that induce these events are the activation of the complement cascade, the release of cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and the increased expression of adhesion molecules. This inflammatory events are relevant in the skin, lung, and liver and may also play a role in the kidney (1–4). Until now, it has been demonstrated that glomeruli release the cytokines IL-1β and TNF-α after stimulation by endotoxin (5). In addition, in a rabbit model of endotoxemia, the continuous infusion of TNF-α leads to glomerular leukocyte accumulation (6)

How the glomerular leukocyte recruitment is regulated during endotoxemia is incompletely understood. Recently, a class of chemotactic cytokines called chemokines has been characterized. These small cytokines are classified into two families, α and β chemokines, depending on the cysteine residues in their amino acid sequence. The most intensively studied member of the α-chemokine family is IL-8. In the β-chemokine family, the most studied members are monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation normal T cell expressed and secreted), which shows chemotactic activity also for monocytes/macrophages (reviewed in references 7 through 10). The increased formation of several of these chemokines has recently been shown in a variety of kidney diseases, and a role for the infiltration of leukocytes has been described for cytokine-induced neutrophil chemoattractant, IL-8, and MCP-1 by the use of selective antibodies (11–15).

These data strongly suggest that chemokines in general might be of pivotal importance in the regulation of inflammatory cell recruitment in renal disease. Therefore, it is of interest to define their exact function in renal injury and to characterize the mechanisms that regulate their formation and degradation. Recent data reveal that nitric oxide (NO) limits the severity of endotoxin-induced tissue damage and reduces TNF-α synthesis in lipopolysaccharide (LPS)-stimulated macrophages in vitro and prevents endotoxin-induced glomerular thrombosis (16–18).

Therefore, we hypothesized that endotoxemia-induced recruitment of inflammatory cells in the kidney might be medi-
ated by chemokines and studied whether this effect could be modulated by NO. In the present study, we examined the effect of LPS on glomerular formation of RANTES and tested the potential role of L-arginine/NO in this process. Our data show that LPS stimulates RANTES formation in glomeruli, which results in a concomitant increase of monocyte/macrophage infiltration, a pathologic feature that was further increased by inhibition of NO formation.

Materials and Methods

LPS Treatment

Male Wistar rats (100 to 120 g body wt; Charles River Wiga, Sulzfeld, Germany) were injected intraperitoneally (i.p.) with LPS (Escherichia coli type O111:B4; Sigma, Munich, Germany). The doses used were 50, 100, or 250 μg of LPS/100 g body wt.

For in vivo interventional studies, 200 mg/kg body wt L-arginine (Sigma, Munich, Germany) or 30 mg/kg body wt Nω-nitro-L-arginine methylester (L-NAME; Sigma) were given i.p. three times before and after LPS application (Fig. 1). For control experiments, phosphate-buffered saline (PBS) was injected i.p., and animals were left untreated or treated with four applications of L-arginine or L-NAME i.p. at the same time intervals.

Morphology

Kidney samples were fixed in 4% buffered formalin and embedded in paraffin. For light microscopy, 4-μm-thick sections were stained with periodic acid-Schiff. Immunohistochemistry was performed with ED-1 antibodies (Chemicon International, Temecula, CA) to determine glomerular monocytes/macrophages. Alkaline phosphatase antialkaline phosphatase (APAAP) (Caman, Wiesbaden, Germany) was used as the detection system.

Nitrite and RANTES Measurements in Isolated Glomeruli

Glomeruli from freshly harvested kidneys were isolated by a sieving technique as described elsewhere (19) and incubated in phenol red-free Dulbecco’s modified Eagle’s medium containing 4.5 g of glucose per liter, supplemented with 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum, 100 μg of ampicillin, and 100 μg of streptomycin per milliliter (Life Technologies) for 48 h.

Nitrite (NO2⁻/NO3⁻) released into the supernatant of incubated glomeruli was determined with the Griess reagent (1 k sulfonamide, 0.1 M 2-mercaptoethanol. Twenty micrograms of total RNA was electrophoresed through a 1.2% agarose gel in running buffer (2.2 M formaldehyde, 0.02 M 3-(N-morpholino)-propanesulfonic acid, and 1 mM ethylenediamine tetra-acetic acid (EDTA)). The RNA was transferred onto Zetabind membranes (Cuno, Meriden, CT), prehybridized after ultraviolet cross-linking at 42°C for 24 h in 5× saline-sodium phosphate-EDTA (SSPE) (20× SSPE: 3 M NaCl, 0.2 M Na2HPO4, and 0.02 M EDTA), 5× Denhardt’s solution (50× Denhardt’s: 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin), 50% formamide, 0.1% sodium dodecyl sulfate, and 0.1 mg/ml sheared salmon sperm DNA (Sigma, Deisenhofen, Germany). All cDNA fragments were separated from their plasmids in low-melt agarose. The cDNA probes used were an EcoRI–Xhol fragment of the cDNA encoding for murine RANTES (20), an HaeII fragment encoding for murine inducible NO synthase (iNOS) (21), and a 2.0-kb insert of pMCI encoding the murine 18S RNA band. The cDNA fragments were labeled with 50 μCi 32P(deoxyadenosine 5'-triphosphate (3000 Ci/mmol, Amersham, Braunschweig, Germany), using a random priming kit (Appligen, Illkirch, Germany). The membranes were hybridized with 106 cpm probe per ml in hybridization buffer (same buffer as used for prehybridization) for 4 h at 42°C using a rotating drum. After hybridization, blots were washed twice in 2× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 0.5% sodium dodecyl sulfate at 65°C for 45 min. Membranes were stripped with 5 mM Tris-HCl, 0.2 M EDTA, 0.5% sodium pyrophosphate, and 5× Denhardt’s solution for 3 h at 65°C and were subsequently rehybridized with the cDNA probe for the 18S band to account for small loading and transfer variations. Exposed films were scanned with a densitometer (GS 365W, Hoefer Scientific Instruments, San Francisco, CA), and the areas under the curves were integrated by Gaussian integration with the computer program GS 365W (Hoefer). Relative changes in RNA were calculated after assigning hybridization in control lanes a relative value of one. Samples were normalized for the signal intensity of the 18S ribosomal RNA hybridization.

Chemotactic Assay

Supernatants of isolated glomeruli from rats with endotoxemia (250 μg/100 g body wt LPS) were collected and used for the chemotactic assay in a modified Boyden chamber, as described previously (22). For this purpose, human monocytes were isolated by Ficoll gradient centrifugation. Monocyte chemotaxis was determined by counting cells adherent to a semipermeable membrane (Schleicher & Schüll, Dassel, Germany) after staining with hematoxylin and eosin. Additional experiments were performed with an anti-RANTES monoclonal antibody (R&D Systems) added to the supernatants. As demonstrated previously by Western blotting, this commercially available monoclonal antihuman RANTES antibody also recognizes rat RANTES (23).

Experimental Design

In the experimental groups, different doses of LPS (50, 100, or 250 μg/100 g body wt) were used depending on the biological effects that were examined in the in vivo experiments. In general, Northern blotting experiments were performed with 100 μg of LPS/100 g body wt. Regulation of RANTES gene expression by the L-arginine/NO pathway were examined with 50 or 100 μg of LPS/100 g body wt, respectively, whereas experiments in which biosynthesis of ex vivo incubated isolated glomeruli was determined were performed with 250 μg of LPS/100 g body wt.

Group I: Time Kinetic Experiments of Glomerular RANTES mRNA Expression in Endotoxemia

LPS was injected in a dose of 100 μg/100 g body wt i.p., and glomerular RANTES expression was
Experimental Design

LPS i.p. 1 h 2 h 6 h 12 h 24 h
L-arginine or L-NAME i.p.

definition of glomerular RANTES and iNOS expression

Figure 1. Experimental design. Kidneys were harvested for further examination at 1, 2, 6, 12, or 24 h after intraperitoneal (i.p.) application of lipopolysaccharide (LPS) in rats. In some experiments, L-arginine or N^G^-nitro-L-arginine methylester (L-NAME) was injected intraperitoneally three times before and once after LPS application.

determined at 1, 2, 6, 12, and 24 h thereafter (Figure 1). Control animals were injected with PBS and examined at the same time intervals.

Before nephrectomy, kidneys were perfused with at least 150 ml of PBS until the effluent was clear, kidney slices were harvested for morphological examinations, and glomeruli were isolated by fractional sieving for isolation of glomerular RNA and Northern blotting. At each time point five animals were examined. The experiments were repeated twice.

Group II: Regulation of Glomerular RANTES mRNA Expression by the L-Arginine/NO Pathway in Endotoxemia. To examine the mechanisms regulating RANTES expression, animals were pretreated with L-arginine or L-NAME. LPS (50 or 100 μg/100 g body wt) was given i.p., and the animals were sacrificed 6 or 12 h later. L-Arginine or L-NAME was given three times before and once after LPS application (Figure 1). Blood was drawn, kidneys were perfused, and glomeruli were isolated and pooled as described above. In addition, kidney slices were fixed for light microscopy. RNA was extracted from isolated glomeruli and used for Northern blotting. Six animals were studied in each group, and each experiment was repeated three times.

Group III: Regulation of Glomerular RANTES Protein Biosynthesis and Nitrite Release by the L-Arginine/NO Pathway in Endotoxemia. In additional control and LPS-treated rats (250 μg/100 g body wt) with or without L-arginine or L-NAME application, kidneys were harvested after 12 h without prior perfusion. Glomeruli were isolated from each kidney separately under sterile conditions and incubated in phenol red-free Dulbecco’s modified Eagle’s medium for 48 h. After 48 h, NO_2^-/NO_3^- release and RANTES biosynthesis were measured in the supernatants. Six animals were studied in triplicate in each group.

Group IV: Chemotactic Assay. In additional experiments, glomeruli were isolated from kidneys 12 h after in vivo LPS (250 μg/100 g body wt) application under sterile conditions. Glomeruli were incubated for 48 h in culture medium. At the end of the 48-h incubation period, supernatants were collected and stored at −20°C until chemotactic activity was examined in a modified Boyden chamber as described above. Six LPS-treated animals and four control animals were examined.

Statistical Analyses

Results are expressed as the mean ± SEM. For statistical comparison, the Mann–Whitney U test was performed. For multiple group comparisons, the nonparametric Kruskal–Wallis test was used. P values <0.05 were considered statistically significant. For Northern blot analysis, an increase or decrease of RNA expression as determined by laser densitometry of at least 25% was considered significant (P < 0.05) and analyzed further with the Kruskal–Wallis test.

Results

RANTES and iNOS Expression in Isolated Glomeruli of Rats with Endotoxemia

RANTES mRNA expression in isolated glomeruli increased with time after in vivo LPS application and reached a maximum at 12 h (Figure 2). L-Arginine treatment significantly reduced glomerular RANTES expression in isolated glomeruli when examined 12 h after LPS application (Figure 3). The reduction of RANTES expression after L-arginine was approximately 80% (P < 0.01).

In contrast, L-NAME significantly increased (P < 0.01) glomerular RANTES expression at 6 and 12 h when the dose of LPS was low (50 μg/100 g body wt) (Figure 4). High doses of LPS given in vivo obviously increased glomerular RANTES expression maximally, which could not be stimulated further by L-NAME treatment. L-Arginine treatment further increased iNOS expression in glomeruli of LPS-treated animals (P < 0.05) (Figure 5). L-NAME pretreatment, however, did not significantly reduce glomerular iNOS mRNA expression (Figure 5).

RANTES Protein

RANTES protein released into the supernatant of isolated glomeruli from rats with endotoxemia was significantly increased (P < 0.01), as determined by enzyme-linked immunosorbent assay (Figure 6). L-Arginine significantly (P < 0.05) reduced RANTES protein released into the supernatant of isolated glomeruli of rats with endotoxemia (Figure 6).

Nitrite/Nitrate Biosynthesis

NO_2^-/NO_3^- released into the supernatants of isolated glomeruli was significantly increased (P < 0.01) after in vivo LPS infection.

Statistical Analyses

Results are expressed as the mean ± SEM. For statistical comparison, the Mann–Whitney U test was performed. For multiple group comparisons, the nonparametric Kruskal–Wallis test was used. P values <0.05 were considered statistically significant. For Northern blot analysis, an increase or decrease of RNA expression as determined by laser densitometry of at least 25% was considered significant (P < 0.05) and analyzed further with the Kruskal–Wallis test.
application compared with controls (Figure 6). In vivo l-arginine treatment further increased ($P < 0.01$) the NO$_2$/NO$_3$ release into the supernatant of isolated glomeruli of rats with endotoxemia (Figure 6). The increased NO$_2$/NO$_3$ synthesis was not inhibited significantly by l-NAME treatment in ex vivo incubated glomeruli, probably due to pharmacodynamic reasons (Figure 6).

**Histology**

LPS-treated animals showed glomerular enlargement and eventually glomerular thrombosis. The number of infiltrating ED-1-positive glomerular cells significantly increased at 12 h after LPS application (100 µg of LPS/100 g body wt), from 0.8 ± 0.2 (control) to 5.2 ± 1.4 (LPS) ($P < 0.01$). This increase in ED-1-positive cells was significantly inhibited by l-arginine treatment of endotoxemic rats to 2.8 ± 1.6 ($P < 0.05$). Pretreatment of rats with l-NAME significantly increased ($P < 0.01$) the number of ED-1-positive glomerular cells 12 h after application of a low dose of LPS (50 µg/100 g body wt) (Figure 7). l-NAME did not result in a further increase in the number of ED-1-positive glomerular cells in rats given a high dose of LPS (100 µg/100 g body wt for 12 h).

**Chemotaxis**

The conditioned media of isolated glomeruli of endotoxemic rats were chemotactic for human monocytes (Table 1). Chemotactic activity of the supernatants was inhibited by approx-
Figure 6. The release of RANTES or NO₂/NO₃ into the supernatant of isolated glomeruli from rats 12 h after i.p. LPS injection (250 μg/100 g body wt) and cultured for an additional 48 h in vitro. *P < 0.05 versus controls; **P < 0.01 versus controls; ***P < 0.05 versus untreated LPS rats.

Discussion

The present experiments demonstrate that the chemokine RANTES is expressed and secreted in isolated glomeruli of rats with endotoxemia. The increased glomerular RANTES formation in this model is associated with enhanced recruit-
mament of glomerular macrophages. The RANTES expression is reduced by L-arginine, which also increased the glomerular release of NO\textsubscript{2}/NO\textsubscript{3} and inhibited the infiltration of macrophages. The results, therefore, suggest a new role of the L-arginine/NO pathway in the regulation of RANTES.

In in vitro experiments, we have demonstrated previously that LPS induced the chemokine RANTES in glomerular mesangial cells in culture (24). We now demonstrate that RANTES expression is also increased in isolated glomeruli after in vivo application of LPS. This enhanced glomerular RANTES expression is associated with an increased number of glomerular macrophages, as shown by an increased number of ED-1-positive cells. Furthermore, glomeruli isolated from rats with endotoxemia released RANTES protein into the supernatant when incubated in vitro for an additional 48 h. The culture supernatant of the ex vivo incubated glomeruli was chemotactic for monocytes as demonstrated in a modified Boyden chamber assay, and this increased chemotactic activity could be blocked by an anti-RANTES antibody. These experiments clearly demonstrate that RANTES is expressed and secreted in glomeruli of rats with endotoxemia and that RANTES isolated from these LPS-stimulated glomeruli is chemotactic for monocytes.

NO has previously been shown to protect mice from endotoxin shock, to reduce TNF-\(\alpha\) expression in macrophages after LPS stimulation, and to inhibit glomerular thrombosis in endotoxemia (17,18). We therefore examined whether NO might also regulate glomerular RANTES expression and inflammatory cell infiltrate in in vivo endotoxemia.

In vivo LPS application is known to increase glomerular iNOS expression and NO synthesis in endotoxin-treated rats. In our study, this increased glomerular iNOS expression in rats with endotoxemia has been further increased by pretreating rats with L-arginine, which is consistent with data from in vitro experiments in which NO was shown to increase its own biosynthesis in IL-1\(\beta\)-stimulated glomerular mesangial cells (25). Pretreatment with L-arginine was also associated with an increased glomerular NO biosynthesis, as demonstrated by the release of increased amounts of NO\textsubscript{2}/NO\textsubscript{3} into the supernatant of isolated glomeruli. This increased NO biosynthesis was associated with reduced glomerular RANTES formation. Conversely, an unspecific inhibitor of NO synthase, L-NAME, significantly increased glomerular RANTES expression and also significantly increased the number of infiltrating glomerular macrophages, at least in animals given a low dose of LPS.

The results of our experiments and those of other researchers that NO might be beneficial in endotoxemia are in contrast to other experiments, in which NO was found to have deleterious effects in endotoxin shock. These experiments, however, examined contraction of vascular smooth muscle cells or hemodynamic parameters and generally found that NO synthesis inhibitors improved vascular functions in endotoxemia (26–28). Thus, there might be differences in the regulation of early gene products and systemic hemodynamic parameters by the L-arginine/NO pathway in endotoxemia.

The mechanisms by which NO reduced glomerular RANTES expression and hence the number of infiltrating glomerular macrophages have not been elucidated in the present in vivo experiments. In in vitro experiments, however, it has been recently shown that NO reduced MCP-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibition of NF\(\kappa\)B activation, probably mediated by an NO-induced inhibition of oxygen radical formation (16, 29–33).

We have not determined whether additional NO synthases other than iNOS are also stimulated by L-arginine application in the experimental model of endotoxemia and which therefore might also be responsible for reduced RANTES expression. LPS is known to stimulate the expression and activity of iNOS. However, it cannot be concluded from these experiments whether L-arginine is metabolized exclusively by iNOS in endotoxemia. It may be that cytokines released from LPS-stimulated glomerular mesangial cells or infiltrating inflammatory cells stimulate glomerular endothelial cells, which then metabolize L-arginine by the constitutively expressed endothelial NO synthase. Additionally, it has been demonstrated in in vitro experiments that endothelial cells release NO for a prolonged period in response to TNF-\(\alpha\), indicating that endothelial cells express both a constitutive and an inducible NO synthase activity (34). The cross-talk between glomerular mesangial and endothelial cells with respect to the L-arginine metabolism in endotoxemia by the various NO synthases must be examined further.

The effect of L-arginine on glomerular RANTES expression and glomerular infiltration of ED-1-positive cells may be attributed to more than an increased glomerular NO formation. The role of L-arginine in renal disease has recently been reviewed (35,36). Beneficial effects of L-arginine administration have been demonstrated in obstructive nephropathy, in subtotal renal ablation, in systemic hypertension, in cyclosporin A nephrotoxicity, and in experimental diabetes (37–39). L-Arginine depletion was shown to exacerbate nephrotoxic serum nephritis (40). Most of the beneficial effects of L-arginine administration have been attributed to improved glomerular hemodynamics by counteracting vasoconstriction, inhibition of thrombosis, or oxygen radical formation. However, it is not yet completely understood by which mechanism L-arginine reduced the number of infiltrating macrophages in obstructive nephropathy or in puromycin aminonucleoside-induced nephrosis. It became evident that the number of infiltrating macrophages is reduced not only because of the beneficial effects of L-arginine on renal hemodynamics, but also because of some

### Table 1. Chemotactic assay\(^a\)

<table>
<thead>
<tr>
<th>No. of monocytes per high-power field</th>
<th>control</th>
<th>LPS</th>
<th>LPS + RANTES antibody (in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 ± 0.3</td>
<td>25 ± 2.4(^b)</td>
<td>10 ± 1.8(^c,d)</td>
</tr>
</tbody>
</table>

\(^a\) LPS, lipopolysaccharide; RANTES, regulated upon activation normal T cell expressed and secreted.

\(^b\) \(P < 0.01\) versus control.

\(^c\) \(P < 0.05\) versus control.

\(^d\) \(P < 0.05\) versus untreated LPS.
additional mechanisms induced by the application of L-arginine. In a model of unilateral ureteral obstruction, L-arginine treatment also reduced collagen α1 (IV) mRNA expression and synthesis in the kidney cortex (41).

L-Arginine is metabolized not only by NO synthases, but also by arginase to form L-ornithine and by arginine decarboxylase to form the end product agmatine (42,43). L-Ornithine might have effects on cell proliferation and collagen synthesis. The role agmatine might play in renal pathophysiology has not been examined in detail; however, agmatine may be an endogenous inhibitor of other L-arginine pathways (43,44).

These data on the beneficial effects of L-arginine in some experimental models of renal disease are in conflict with recently published studies in which NO synthesis inhibition or restriction of dietary L-arginine intake were found to have beneficial effects in a model of experimental mesangial proliferative glomerulonephritis and to reduce mesangial cell lysis (45). The differences in the effects of L-arginine supplementation or restriction on the number of infiltrating inflammatory cells or the lysis of mesangial cells in the different models of renal inflammation are, at present, not clearly understood.

In conclusion, the present experiments demonstrate an increased RANTES expression in isolated glomeruli in endotoxemia and a negative regulation of glomerular RANTES expression by the L-arginine/NO pathway. These studies offer new evidence that the L-arginine/NO pathway may have beneficial effects in renal inflammatory diseases, which are not limited to the improvement of glomerular or systemic hemodynamics, but might also be important in the regulation of glomerular chemokine expression and the subsequent infiltration of inflammatory cells.

Acknowledgments

This work was supported by Grant DFG Th 343/8-1 from the Deutsche Forschungsgemeinschaft. The authors gratefully acknowledge S. Roscher and U. Kneissler for skillful technical assistance.

References


