Local Production of Inflammatory Mediators in an Experimental Model of Acute Obstructive Pyelonephritis

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To investigate bacterial growth and inflammatory mediator release in the early stage of the immune response, a unilateral acute ascending pyelonephritis was induced in rats by intra-bladder inoculation of Escherichia coli. The infected left kidney showed a significant bacterial proliferation, local production of interleukin (IL)-6 and IL-8 as detected by immunocytochemistry, and extensive destruction of renal parenchyma associated with impressive leukocyte recruitment. Inducible and constitutive nitric oxide synthases (NOS) were locally expressed, and a time-dependent increase in urinary secretion of nitric oxide (NO) was seen that could be blocked by N^G^-monomethyl-L-arginine. However, there was a discrepancy between the NO profile in the kidney and urine. The results demonstrate that in the early stage of acute pyelonephritis kidney tubules participate actively in the local host response by producing important inflammatory mediators and that urinary NO levels are not suitable for predicting renal NOS activity.

Preventing or limiting renal damage, including scar formation and deterioration in renal function, should be a primary goal in the therapy of pyelonephritis. Previous studies indicated that early events in the acute stage of pyelonephritis greatly influence the pathologic outcome of this disease [1–5]. Data suggest that inflammatory mediators such as cytokines, toxic metabolites, and enzymes released from leukocyte infiltrates are in part responsible for structural damage to the parenchyma leading to renal scarring [4, 6]. In acute pyelonephritis, the inflammatory response is mostly localized to the site of infection and not systemic. Several studies have shown that kidney cells can produce local inflammatory mediators in response to invading pathogens [7, 8]. Recent clinical and experimental investigations suggest that both the urinary excretion and serum level of cytokines are effective markers of renal damage that could be used to monitor the evolution and outcome of the disease [8–12]. However, these results should be interpreted with caution because there is not necessarily a good correlation between cytokine production by uroepithelial cells of the mucosa in response to bacteria (leading to secretion into urine) [13] and cytokines originating from kidney tissues.

To investigate the relationship between bacterial colonization, renal scarring, and expression of inflammatory mediators currently recognized as involved in the early stage of host response in acute pyelonephritis, we used an experimental rat model of acute ascending Escherichia coli pyelonephritis. We followed bacterial dissemination into renal parenchyma and detected, by immunohistochemistry techniques, the renal production of interleukin (IL)-6 and -8, nitric oxide (NO), and leukocyte infiltration. Renal damage, both to the morphology and the ultrastructure (histology), was noted. Biochemical studies included detection of NO production in urine, kidney, and serum. We also used a nonspecific inhibitor of nitric oxide synthases (NOS), N^G^-monomethyl-L-arginine (L-NMMA), to further evaluate the secretion of NO in our model.

Materials and Methods

Pyelonephritis Model

Acute retrograde pyelonephritis was induced in female Sprague Dawley rats weighing 150–200 g as previously described [5]. In brief, animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (0.1 mL/100 g). The left ureter was exposed through a midline incision of the abdominal wall. At the midureter level, a polypropylene suture was passed through the flank into the peritoneal cavity surrounding the ureter. The ligature was left loose in place while an inoculum of 10^8 E. coli (Yale strain) cells of an overnight culture in Mueller-Hinton broth (MHB; Difco, Detroit) was slowly injected into the bladder in a 0.3-mL volume of MHB. Fifteen minutes later, the two arms of the ureteral ligature were tied through the left flank, and the abdominal wall was closed. Twenty-four hours later, the animals were anesthetized and the ligature was cut from the outside and carefully removed. This procedure produced partial obstruction of urine flow followed by severe unilateral pyelonephritis in the left kidney. Grown under the conditions of this experiment, this E. coli strain expresses fimbriae and has induced pyelonephritis and abscesses in other models of renal infection in the absence of ureteral obstruction [14–17].
Infected animals were divided into two groups. The first group was infected with a single inoculum of bacteria as above, and the second group was infused with bacteria supplemented with 0.5 mM L-NMMA (Calbiochem, La Jolla, CA). Two additional groups were used as controls: normal rats that did not undergo any surgery, and sham-operated rats that had their bladders infused with 0.3 mL of sterile MHB. Except for animals examined 6 h after infection, rats were individually housed in metabolic cages (specifically designed by the manufacturer to minimize fecal and rat chow debris in urine), and urine was collected over a 24-h period under mineral oil, and then centrifuged, aliquotted, and stored at −20°C for NO assay. Rats had free access to standard rat chow and water throughout the experiment.

Animals were sacrificed at 6, 24, and 48 h after induction of pyelonephritis. Under anesthesia, urine was collected by bladder puncture for bacterial enumeration, then rats were sacrificed by decapitation and blood was collected. Serum obtained by centrifugation was stored at −20°C. The abdomen was opened and both kidneys were removed aseptically then weighed and cut in half: One half was divided into two pieces—one part was processed for histopathology and the second part for immunohistochemistry. The second half of each kidney was immediately homogenized for bacterial enumeration. Some homogenates were frozen at −20°C for further NO assay.

The kidney cortex, medulla, and papilla were separated by dissection, and each part was homogenized in saline and serially diluted before culture (in triplicate) on MacConkey’s agar (Quélab, Montreal). After plates were incubated aerobically for 18–20 h at 37°C, colonies were counted and results expressed as log colony-forming units (cfu) per gram. Urine was also plated and colony counts were expressed as log cfu per milliliter.

Urine, serum, and kidney homogenate samples were assayed for the stable end products of NO: nitrates (NO$_3$) and nitrites (NO$_2$). The samples were diluted 1:10 with saline and centrifuged at 16,000 g (Eppendorf microcentrifuge) for 5 min at room temperature. Aliquots (100 μL) were incubated with Aspergillus nitrate reductase in the presence of β-NADPH (Sigma) to reduce NO$_3$ to NO$_2$. The resulting samples were assayed for total NO$_2$ content by the Griess reaction [18, 19]. In brief, the aliquots were incubated with 200 μL of 1% sulfanilamide and 200 μL of 0.1% N-1-naphthylenediamine dihydrochloride in 2.5% phosphoric acid at room temperature for 1 h. Absorbance at 540 nm was measured in a microplate reader. Standard NO$_2$ calibration was done using NaNO$_2$.

Results were compared using one-factor analysis of variance for repeated measures. Comparison of group means was done by Fisher’s protected least significant difference test. P < .05 was considered statistically significant.

**Results**

Kidney morphology. The macroscopic appearance of the left kidney from infected, sham-operated, and normal noninfected rats appeared similar 24 h after infection, although infected kidneys looked more ischemic (pale) than those sham operated. The left kidney from the infected and sham-operated rats also appeared slightly enlarged compared with right kid-
neys in the same groups. A high percentage of pyelonephritic kidneys displayed numerous abscesses over the cortex surface 48 h after infection.

**Bacteriologic results.** Table 1 shows bacterial counts in urine, whole kidney, and in different kidney regions. Six hours after infection, bacteria were detected only in urine (1.7 ± 1.1 log cfu/mL). By contrast, kidney homogenates 24 and 48 h after infection had considerable bacteria. In fact, bacterial counts increased in a time-dependent manner in urine, cortex, medulla, and papilla. Although similar cfus per gram were observed at 24 h in every part of the kidney, a predominant growth occurred in the cortex and medulla at 48 h.

**Immunohistochemical staining for E. coli.** Detectable labeling only appeared 24 h after infection in kidney sections and was characterized by sparse *E. coli*–positive staining (data not shown). In contrast, significant staining was seen at 48 h in the lumen, interstitium, and tubules of the cortex, medulla, and papilla (data not shown). Some nephrons were heavily labeled throughout the papilla, medulla, and cortex, but others had little labeling. Ultrastructural observations revealed immunogold labeling of *E. coli* within the tubular cells (figure 1A). Immunoreactive bacteria were also found within phagocytes in the tubular lumen (figure 1B). Unidentified structures were found in the cytoplasm of phagocytes, suggesting the presence of digested bacterial material. In areas of severe inflammation, polymorphonuclear cells (PMNL) with enlarged vacuoles containing numerous immunoreactive bacteria were also seen (figure 1C, 1D) as was cellular debris. Electron microscopy of infected kidneys at 48 h showed impressive leukocyte recruitment and extensive areas of tissue injury and tubular necrosis. Normal controls and sham-operated and infected kidneys did not show any immunoreactivity 6 h after inoculation (data not shown). Staining specificity was confirmed by positive control with isolated bacteria (figure 1F).

**Immunohistochemical staining for IL-6 and IL-8.** Kidney sections were labeled separately for IL-6 and IL-8 at 6, 24, and 48 h after infection. Intense tubular staining was seen in cortical and medullary areas as early as 6 h after bacterial challenge for IL-6 (figure 2A, 2B) and IL-8 (figure 2D, 2E). The same pattern persisted at 24 and 48 h for IL-6 (data not shown), whereas staining for IL-8 remained unchanged at 24 h but declined significantly 48 h after infection (figure 2G, 2H). The staining for IL-8 was limited to proximal and collecting tubules in the cortex and medulla, and only a few tubules were positive. No significant IL-6 and IL-8 staining was seen in the papilla at any time (figure 2C, 2F, 2I), and there was only weak immunoreactivity in normal kidney for both cytokines (data not shown). Tubular staining for cytokines was completely blocked by overnight preincubation of anti-human IL-6 or IL-8 with human recombinant IL-6 or IL-8 cytokines, respectively (data not shown).

**Immunohistochemical staining for monocytes and macrophages.** Kidney sections labeled for monocyte/macrophage detection showed ED-1–positive cells 24 h after infection. At that time, a few macrophages were localized in the cortex (figure 3B), medulla (figure 3E), and papilla (figure 3H). At 48 h after infection, ED-1–positive cells increased massively in all parts of the kidney (figure 3C, 3F, 3I). These macrophages mainly surrounded glomeruli and proximal, distal, and collecting tubules. No ED-1–positive cells were detected in kidney sections from normal controls (figure 3A, 3D, 3G), sham-operated controls, and animals sacrificed 6 h after infection (data not shown).

**Immunohistochemical localization of NOS.** Normal kidney sections showed immunoreactivity for both cNOS (figure 4A) and iNOS (figure 4B) isoforms. Labeling was mainly expressed in the proximal tubules. cNOS labeling was seen as a dark line bordering the proximal tubule lumen as shown in the cortex (figure 4A, 4C, 4E). There were numerous dilated immunoreactive proximal tubules near glomeruli of infected kidneys 24 h after infection (figure 4C, 4D) but not in normal uninfected controls (figure 4A, 4B). A similar observation was noted 6 h after infection (data not shown). cNOS immunoreactivity was also evident in kidneys from sham-operated rats at 24 h but iNOS was not (data not shown). However, 48 h after infection, cNOS staining decreased significantly in infected and sham-operated rats and was limited to a few immunoreactive tubules (figure 4E). iNOS immunoreactivity was also observed at the tubular structure near glomeruli, but unlike cNOS, iNOS staining appeared to be more intracellular as shown by tubule immunoreactivity in the cortex of normal controls (figure 4B). Kidney sections 24 h after infection (figure 4D) showed an increased number of tubules labeled for iNOS as compared with the normal control (figure 4B). The heavy labeling persisted until 48 h after infection (figure 4F), and tubules appeared more dilated than at the previous time point. iNOS staining with sham-operated rats was never as evident as in infected rats throughout our study (data not shown). Histochemical detection of NADPH-dependent diaphorase performed in parallel with the cNOS staining in the papilla of consecutive kidney sections revealed a good correlation between the distribution pattern of NOS and NADPH-diaphorase staining (data not shown).

### Table 1. Bacteriologic studies in rat kidneys.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time after infection (hs)</th>
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<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Urine</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td>Papilla</td>
<td>ND</td>
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<td>Medulla</td>
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<tr>
<td>Cortex</td>
<td>ND</td>
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<td>Whole left kidney</td>
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**NOTE.** Data are at 6, 24, and 48 h after induction of ascending obstructive *Escherichia coli* pyelonephritis. ND, below limit of detection.

a Data are log 10 cfu/mL.

b Data are log 10 cfu/gram of tissue.
Figure 1. Electron micrograph of kidney 48 h after infection shows immunogold labeling of *Escherichia coli*. All magnifications indicated are before reduction for publication. A, Kidney tubular cells appear to have integrated bacteria (arrow). Gold particles appear as black dots. ×19,200. B, *E. coli* (arrow) in phagocytic vacuoles in lumen (L) of tubules (T). Undefined structures (probably degenerated bacterial material) were labeled in cytoplasm of phagocytes. ×9600. C, Recruited polymorphonuclear cells (P) and cellular debris. Note enlarged vacuole containing constellation of immunoreactive *E. coli*. ×8000. D, Numerous labeled *E. coli* in large vacuole. ×32,000. E, Isolated *E. coli* used as positive control. ×32,000. F, Lack of labeling after anti-*E. coli* preabsorption with *E. coli*, thereby confirming specificity of labeling. ×50,000.
Figure 2. Immunohistochemical analysis of cytokine production within kidney after staining with mouse monoclonal antibodies (IgM) to rat interleukin (IL)-6 and -8 and development by indirect immunoperoxidase method. 6 h after infection, staining for IL-6 (A, B) and IL-8 (D, E) in tubules in cortex (A, D) and medulla (B, E). 48 h after infection, extinction of immunoreactivity for IL-8 in cortex (G) and medulla (H). No significant immunoreactivity in papilla (C, F, and I). Original magnification ×105.

Measurement of NO. NO production in the left kidney was detected by measurement of total nitrite levels in kidney homogenates (figure 5A). A significant increase was observed from 24 h after infection in the *E. coli*-infected and sham-operated rats ($P < .01$ compared with control levels at 0 h). NO levels increased further at 48 h in the infected animals but returned to normal in the sham-operated rats ($P < .01$ infected vs. sham). L-NMMA could not prevent NO production in kidney tissues.

There was no significant elevation in urinary NO secretion at 6 and 24 h in infected and sham-operated animals compared with time 0 (figure 5B). By contrast, NO at 48 h increased dramatically in these 2 groups ($P < .01$). L-NMMA abrogated NO release in urine, as levels similar to control values were obtained in the *E. coli* plus L-NMMA treated animals ($P < .01$ vs. untreated infected animals).

NO levels increased significantly ($P < .01$) in serum early after infection with *E. coli* (145% and 183% of the control level at 6 h and 24 h, respectively). Values similar to preinfection levels were observed thereafter. Sham-operated animals did not have any increase. Injection of L-NMMA to infected animals totally blocked the NO increase in serum ($P < .01$).

Discussion

Acute pyelonephritis remains one of the most frequent nosocomial infectious diseases. Although rarely responsible for acute renal failure in the absence of underlying kidney disease, renal infection may induce strong inflammation, transient impairment in renal function, and scar formation. Although *E. coli* virulence factors such as fimbriae have been associated with bacterial colonization and disease, it is well recognized that bacteria alone are not responsible for the severity of pyelone-
Figure 3. Immunohistochemical analysis of ED-1–positive monocytes and macrophages. Kidney sections stained with mouse monoclonal antibodies (IgM) to rat monocytes and macrophages and developed by indirect immunoperoxidase method. Normal sections lack ED-1–positive cells: cortex (A), medulla (D), and papilla (G). 24 h after infection, there are few but significant numbers of ED-1–positive cells (arrowhead) in cortex (B) and medulla (E) but not in papilla (H). At 48 h after infection, numerous ED-1–positive cells in cortex (C), medulla (F), and papilla (I). Original magnification \( \times 125 \).
Figure 4. Immunohistochemical analysis of constitutive (c) NOS and inducible (i) NOS. Kidney sections stained with rabbit polyclonal antibodies to cNOS and iNOS. Weak staining (arrowhead) for cNOS (A) and iNOS (B) in sections from normal control rat. 24 h after infection, significant staining for cNOS (C) and iNOS (D). At 48 h, loss in immunoreactivity for cNOS (E) but persistent staining for iNOS (F). Original magnification ×150.
Inflammatory Mediators in Acute Pyelonephritis

We found gradual proliferation of bacteria from urine to all parts of the kidney, though not all nephrons were labeled to the same extent. Very early IL-8 expression preceded PMNL and monocyte recruitment, suggesting high sensitivity to infection and probable chemotactic influence of this cytokine in our model. We found concomitant and time-dependent increases in monocyte and iNOS expressions and in NO levels in tissues. These coincided with maximal tissue injury, were not induced in sham-operated animals, and could not be blocked (NO release) by L-NMMA. By contrast, cNOS expression in tissue and NO in urine most likely reflected surgical procedures, and NO could be prevented by L-NMMA. Strong IL-6 expression occurred throughout the experiment and may have reflected stress from both infectious and noninfectious origin. Therefore, a dynamic interaction of microbiologic, immunologic, and mechanical factors characterized the pathogenesis of ascending pyelonephritis.

Our model of infection reproducibly induced severe pyelonephritis in 100% of the rats. Temporary ureteral obstruction was necessary to initiate ascending infection and bacterial multiplication in the left kidney, but once infection was established, it progressed despite removal of the obstruction as in other studies [2, 3, 5, 20]. Pathogens were localized in lumen, tissue, and interstitium after 48 h, in accordance with observations by Fussell and Roberts [21]. Colonization of the cortex and medulla was predominant, which also corresponded to the major sites of IL-6 and IL-8 expression. Of interest, not all of the nephrons were labeled to the same extent for IL-6 and IL-8, suggesting greater activation in areas of intense bacterial proliferation. However, confirmation of a correlation between areas that simultaneously stained positive for these cytokines and for E. coli needs to be demonstrated through double-labeling immunohistochemistry.

Figure 5.

A

Total nitrite levels in kidney homogenates and urine. Rats were inoculated in bladder with Escherichia coli alone or in combination with N\(^\circ\)-monomethyl-L-arginine (L-NMMA). Sham-operated animals were included as control to evaluate NO production due to ureteral ligature and injection procedures in bladder. Total nitrite was measured using Griess reagent. Data are mean ± SE of 6 rats (analysis of variance). P < .01 vs. basal level at 0 h (***), vs. E. coli + L-NMMA (**), and vs. sham-operated rats (##).

B

Total nitrite levels in urine. Data are mean ± SE of 6 rats (analysis of variance). P < .01 vs. basal level at 0 h (***), vs. E. coli + L-NMMA (**), and vs. sham-operated rats (##).

Figure 5. Total nitrite levels in kidney homogenates and urine. Rats were inoculated in bladder with Escherichia coli alone or in combination with N\(^\circ\)-monomethyl-L-arginine (L-NMMA). Sham-operated animals were included as control to evaluate NO production due to ureteral ligature and injection procedures in bladder. Total nitrite was measured using Griess reagent. Data are mean ± SE of 6 rats (analysis of variance). P < .01 vs. basal level at 0 h (***), vs. E. coli + L-NMMA (**), and vs. sham-operated rats (##).
In the present study, both IL-6 and IL-8 were secreted early, but only IL-8 expression decreased as infection progressed. IL-8 is well known as a powerful chemoattractant for leukocytes [32]. Cytokine cascades often characterize host response, so that transient appearance of successive chemokines might contribute both to PMNL and monocyte recruitment. In fact, basal levels of both cytokines were expressed in infected, sham-operated, and uninfected animals, suggesting endogenous secretion in normal conditions. This is supported by in vitro studies and observations in human kidneys that may produce cytokines constitutively or upon stimulation by bacterial endotoxin or other cytokines [30, 33–36]. By responding to endotoxin, renal tubular cells could rapidly induce the generation of IL-8 and PMNL recruitment through increased expression of the CD11/CD18 adhesion molecules [37], with ensuing respiratory burst and generation of highly reactive oxygen radicals and secretion of collagenase, elastase, and \( \beta \)-glucuronidase. The latter are toxic to bacteria and can damage renal tubules [38, 39].

Because both PMNL and monocytes are crucial components of acute inflammation, histopathology and cell labeling were done. At 48 h after infection, kidney tubules showed extensive cell necrosis associated with impressive leukocyte recruitment. Our results corroborate the progressive PMNL recruitment observed by Fussell and Roberts [21]. Monocytes and macrophages, which were recognized by ED-1 antibody [40], may also release toxic components including NO that may form peroxynitrites through reaction with oxygen radicals. In fact, oxidative materials may have induced lipid peroxidation and cell membrane injury [41], facilitating bacterial dissemination into parenchymal cells.

Of particular interest in our study was the NOS expression and NO release. NO may exert a wide range of activities in inflammation and immunity in addition to its regulator role in blood flow and renal function. The types I and III isoenzymes are constitutively expressed (cNOS) and maintain homeostasis through immediate secretion of low NO levels, while the inducible type II NOS (iNOS) synthesizes large amounts of NO under stimulation by cytokines or endotoxin [42, 43]. Both beneficial and detrimental effects have been reported, including microbicidal activity and cytotoxicity to host cells (reviewed in [44, 45]).

The measurement of NO levels in kidney homogenates showed a significant increase in NO secretion both in infected and sham-operated kidneys 24 h after infection. While NO in infected kidneys could be interpreted as a result of exposure to bacterial products, the NO increase in sham-operated animals might result from ureteral obstruction as suggested by others [46–48]. This is consistent with the hypothesis that the increased angiotensin II–mediated vasoconstriction resulting from kidney obstruction would be counteracted by local secretion of vasodilator NO [47, 48]. The decrease in NO levels that was observed in kidney homogenates of sham-operated rats 48 h after surgery supports to that hypothesis. Unlike the findings in sham-operated animals, NO release in infected kidneys increased in a time-dependent manner in parallel with the expression of iNOS. Immunolabeling of kidney sections with anti-cNOS and anti-iNOS PAb showed positive reactivity 24 h after infection for both cNOS and iNOS. iNOS labeling persisted and was further enhanced 48 h after infection, and reduction in cNOS immunoreactivity was evident at the same time. These observations suggest that intrarenal NO secretion beyond 24 h mainly resulted from sustained iNOS activity, while 24-h levels could originate both from iNOS and cNOS, and cNOS would be mostly activated by ureteral obstruction.

Since there is an impressive leukocyte accumulation at the inflammatory sites 48 h after infection, and monocytes/macrophages, neutrophils, and renal tubules may all produce large amounts of NO from iNOS in response to endotoxin and cytokines [49–51], the significant release of NO in the infected kidneys was expected. The NO accumulation at 48 h coincided with the decrease in IL-8 expression by tubular cells. Since IL-8 decreases iNOS activity [43], one can imagine a down-regulatory network for IL-8 that would allow iNOS-dependent bacterial mechanisms to proceed once leukocytes have been recruited. We also noted weak iNOS immunoreactivity in normal nonoperated kidneys, indicating that basal NO production is required for normal renal physiology as suggested by Mohaupt et al. [52].

As expected, there was a striking increase in urinary NO in both infected and sham-operated rats since bladder epithelial cells synthesize iNOS in response to bacterial toxins and as a consequence of inflammation initiated by the trauma caused by needle puncture during inoculation. To determine how much urinary NO reflected renal production, we inhibited the bladder NOS activities with L-NMMA, a structural analogue of L-arginine and a competitive inhibitor of its conversion to NO. Supplementation of \( E. coli \) inoculum with L-NMMA did not significantly reduce kidney NO levels, but urinary secretion was almost totally prevented. Since NO secretion is monitored through nitrite levels, and L-NMMA does not eliminate already formed nitrates, it is obvious that most nitrates measured in urine of untreated infected rats did not originate from kidney cells, but rather came from injured bladder epithelial cells, and that monitoring NO in urine may not necessarily predict accurately the extent of renal NO production and kidney inflammation.

Infected rats also showed an increase in serum NO as early as 6 h and up to 24 h after infection. This increase could be the result of nitrate and nitrite reabsorption from proximal tubules rather than systemic stimulation, since these metabolites are extensively reabsorbed in normal proximal tubules [53], and tubular distension due to urine retention during the 24 h of left ureteral obstruction probably offered additional opportunity for reabsorption.

Our study demonstrates that in the early stage of acute pyelonephritis, kidney tubules participate actively in the host re-
sponse to infection through the production of local inflammatory mediators such as cytokines and NO and the recruitment of inflammatory cells which, in association with bacterial virulence factors, contribute to the histopathology of renal infection. The characterization of the nature and localization of components that participate in the evolution of the pathogenesis process may help establish innovative therapeutic strategies that will combine antibiotherapy and immunomodulator drugs.

Acknowledgments

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References