Neutrophil Recruitment and Resistance to Urinary Tract Infection

Masashi Haraoka,1,a Long Hang,1 Björn Frendéus,1 Gabriela Godaly,1 Marie Burdick,2 Robert Strieter,2 and Catharina Svanborg1

This study examined the role of neutrophils in the antibacterial defense at mucosal infection sites. Urinary tract infection (UTI) was established by injection into the bladder lumen of Escherichia coli 1177, a fully virulent clinical isolate. Infection of C3H/HeN (lpsa, lpsb) mice recruited neutrophils into the urinary tract, and bacteria were cleared from kidneys and bladders. The neutrophil response was absent in C3H/HeJ (lpsa, lpsb) mice, and bacteria persisted in the tissues. Peripheral neutrophil depletion of C3H/HeN mice was subsequently achieved by pretreatment with the granulocyte-specific antibody RB6-8C5. The E. coli–induced neutrophil recruitment was inhibited, as shown by immunohistochemistry and tissue myeloperoxidase quantitation. As a consequence, bacterial clearance from kidneys and bladders was drastically impaired. Antibody treatment of C3H/HeJ mice had only a marginal effect. The results show that neutrophils are essential for bacterial clearance from the urinary tract and that the neutrophil recruitment deficiency in C3H/HeJ mice explains their susceptibility to gram-negative UTI.

The resistance of human hosts to microbial attack is astonishing. Health is maintained in the midst of a seemingly overwhelming microflora. This is especially apparent at mucosal surfaces, where most microbes first encounter their hosts [1]. To meet the massive microbial challenge and defend the integrity of underlying tissues, specific immune functions and innate host defenses have converged at these sites [2]. Specific immunity can be particularly effective in preventing infections by pathogens with which the host has had prior experience, but the nonspecific or innate defenses seem to be at least as important in preventing mucosal infections [3–8].

This study examined the antibacterial defense of the urinary tract. Early studies suggested that innate mechanisms might be more important than specific immunity for the early resistance to urinary tract infection (UTI) [3, 5]. Mice with genetic defects in specific immunity (nude, scid, or xid mice) were as resistant to UTI as their immunocompetent littermates, but C3H/HeJ mice were highly susceptible [5–9]. C3H/HeJ mice are designated as lipopolysaccharide (LPS) nonresponders but have a general deficiency in cellular responses involving the Toll-like receptor 4 (Tlr4) receptor [10]. Their macrophages react poorly to tumor necrosis factor, vitamin D3, or interleukin (IL)-1 [11, 12], and their mucosal cytokine responses are impaired. We previously showed that C3H/HeJ mice have a neutrophil recruitment deficiency that might explain their inability to clear the infection [8]. Although the technology then available enabled us to postulate a role for neutrophils, we did not have the molecular information and tools to test this hypothesis.

Neutrophils have been discussed as a cause of tissue damage and renal scarring and have been implicated in the antibacterial defense of the urinary tract [13]. Neutrophil recruitment to the infected urinary tract is initiated when bacteria stimulate the epithelial cells to secrete chemokines and express chemokine receptors. In response to the chemotactic gradient, neutrophils leave the circulation, traverse the submucosa, and reach the epithelial barrier that they cross in a chemokine-dependant manner [14]. IL-8 is the main chemokine involved in neutrophil migration across infected uroepithelial cell layers in vitro [14, 15], and macrophage inflammatory protein (MIP)–2 is an important IL-8 homologue in the mouse urinary tract [15].

This molecular information has made it possible to design tools to specifically interfere with the peripheral neutrophil recruitment to infected tissues [14] and to study the effects of neutrophils in the antibacterial defense. This study showed that neutrophils are essential in controlling bacterial persistence in kidneys and bladders and suggests that these phagocytic cells play a primary role in clearing bacteria from the local infection site.

Materials and Methods

Bacteria. Escherichia coli 1177 of serotype O1:K1:H7, was isolated from a child with acute pyelonephritis [16]. The strain is virulent in the mouse UTI model and evokes a strong inflammatory.

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Present affiliation: Department of Urology, Children’s Hospital Medical Center, Boston, Massachusetts, USA.

Reprints and correspondence: Catharina Svanborg, Institute of Laboratory Medicine, Section of Microbiology, Immunology, and Glycobiology, Lund University, Sweden (Catharina.Svanborg@mig.lu.se).

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host response [17]. It expresses P and type 1 fimbriae but is
hemolysin negative. E. coli 1177 was maintained in deep agar stabs
sealed with sterile paraffin, passaged on tryptic soy agar, grown
overnight in static Luria broth and harvested by centrifugation at
1500 g for 10 min. The pellet was resuspended in 0.01 M of PBS
(pH 7.2) to a concentration of 1–2 × 10^6 cfu/mL. The bacterial
concentration was confirmed by viable counts.

Experimental UTI. C3H/HeN and C3H/HeJ mice were bred
in the animal facilities, Department of Medical Microbiology, Uni-
versity of Lund. Female mice were used at age 9–13 weeks. After
anesthesia, mice were infected by intravesical inoculation with E.
coli 1177 (0.1 mL) through a soft polyethylene catheter (outer di-
ameter 0.61 mm; Clay Adams, Parsippany, NJ [18]). The catheter
was immediately removed, and the mice were allowed food and
water ad libitum.

Animals were killed by cervical dislocation after 2, 6, and 24 h
while under anesthesia. Kidneys and bladders were removed and
homogenized in sterile, disposable plastic bags using a tissue
homogenizer (Seward Medical, UAC House, London, UK). The homogenates were diluted in sterile PBS, and 0.1 mL of each dilution was plated on tryptic soy agar.
The number of colonies was scored after overnight culture at 37°C.

Urination was induced by gentle pressure on the mouse abdo-
men, and urine was collected at the urethral orifice into sterile tubes.
Urine samples collected prior to infection were cultured to ensure
infection and were examined for a preexisting neutrophil response. Urine samples collected at 2, 6, and 24 h
postinfection were not used for neutrophil counts and to quantify the
local chemokine response.

Inhibition of neutrophil recruitment. RB6-8C5, a rat immu-
noglobulin G2b (IgG2b) monoclonal antibody (MAb) specific for
murine neutrophils and eosinophils [19, 20] was a kind gift from
Dr. A. Sjöstedt (Umeå University, Sweden), Dr. W. Conlan (Tru-
deau Institute, Saranac Lake, New York), and Dr. R. Coffman
(DNAX Research Institute, Palo Alto, California). MAb RB6-8C5
was purified from hybridoma supernatants by ammonium sulfate
precipitation or by Protein G-Sepharose (Pharmacia Biotech, Upp-
sala, Sweden). The concentration of immunoglobulin from the pu-
rifed MAb was determined by ELISA. The MAb or control rat
IgG (0.25 mg diluted in 0.5 mL of pyrogen-free saline) were injected
intraperitoneally into mice 24 h and again 2 h before bacterial
inoculation.

Polyclonal rabbit antimurine MIP-2 antibodies were generated
by immunization of rabbits with murine MIP-2 (R&D Systems,
Minneapolis) [21, 22]. Mice were injected intraperitoneally with
anti-MIP-2 antiserum or preimmune serum in 0.1% saponin in
saline (TBS-Sap), alkaline phosphatase–conjugated goat anti-rab-
bbit immunoglobulins (DAKO, Copenhagen, Denmark) were added
at a 1 : 50 dilution in TBS-Sap and left to incubate for 60 min at
room temperature in a moist chamber. After washes in TBS-Sap
the Fast-Red substrate containing levamisole (DAKO) was pre-
pared according to the manufacturer’s recommendations, added to
the samples, and left to incubate for 20 min at room temperature.
The samples were thereafter washed in TBS (pH 7.6) and coun-
terstained with Mayer hematoxylin (KEBO Laboratories, Stock-
holm, Sweden) for a few minutes, washed in distilled water, and
mounted with Mount-quick “AQUEOUS” (Daido Sangyo, Tokyo,
Japan). The samples were investigated under a light microscope
(Microphot, Nikon, LRI instrument AB).

Statistical analysis. Differences between samples were analy-
ed with the Mann-Whitney U-test (2-tailed) and the Spearman
rank correlation test. Differences were considered significant for
P < .05.

Results

Difference in bacterial clearance between C3H/HeN and C3H/
HeJ mice. UTI was established by transurethral injection of E.
coli 1177. The bacterial persistence in kidneys and bladders
was determined by viable counts on tissue homogenates from
mice killed at different times after inoculation (figure 1). Most
of the inoculum was cleared from the kidneys of C3H/HeN
mice during the first 24–48 h, with the greatest reduction in
viable counts occurring between 6 h and 24 h. C3H/HeJ mice,
in contrast, were unable to clear the infection. Instead of a decrease during 6–24 h, stationary bacterial numbers were observed (figure 1).

The difference in bacterial clearance between C3H/HeN and C3H/HeJ mice was most pronounced in the kidneys. The bladder counts were higher in C3H/HeN than in C3H/HeJ mice ($P<.05$), but the difference was smaller than in the kidneys, and the maximum difference occurred at 6 rather than 24 h (figure 1).

**Difference in neutrophil recruitment between C3H/HeN and C3H/HeJ mice.** The neutrophil response to infection was quantified in urine samples obtained at various times after inoculation (figure 2A). Prior to infection, there were no neutrophils in urine samples from C3H/HeN or C3H/HeJ mice, but after 2 h, urine neutrophil numbers increased in C3H/HeN mice, reached a peak by 6 h, and remained elevated at 24 h after inoculation. There was a marked difference in neutrophil recruitment between the C3H/HeN and C3H/HeJ mice. Urine neutrophil numbers remained low at all times after inoculation of the C3H/HeJ mice (figure 2A).

Neutrophils in tissue sections stained with hematoxylin and eosin are shown in figure 2B. Sections stained with monoclonal anti-neutrophil antibodies are shown in figure 3. Few neutrophils were seen in kidneys of C3H/HeN mice after 2 h (data not shown), but by 6 h an influx of neutrophils had occurred (figure 2B, figure 3). Large numbers of neutrophils were scattered throughout the renal tissue and adjacent to the pelvic epithelium and were seen crossing the epithelium into the lumen of the renal pelvis (figure 2B, figure 3). By 24 h neutrophil numbers were elevated above the controls, but had decreased, compared with the 6 h time point. The density of neutrophils was lower in bladder tissue sections than in the kidneys (figure 2B), but there was a significant increase in neutrophil numbers in urine. There were very few neutrophils in kidney or bladder sections from C3H/HeJ mice at all the time points after inoculation (figure 2B, figure 3).

**Deficient chemokine response in C3H/HeJ mice.** The neutrophil recruitment deficiency in the C3H/HeJ mice might be caused by a defective chemokine response to bacterial infection. Epithelial cells in the urinary tract mucosa of C3H/HeN mice were recently shown to respond to *E. coli* 1177 infection by producing MIP-2, and blocking MIP-2 was shown to impair neutrophil migration across the epithelial barrier [14]. In this study, tissue sections from C3H/HeJ and C3H/HeN mice were stained for MIP-2 production by using specific antibodies.

MIP-2 was not detected in kidney sections of either C3H/HeN or C3H/HeJ control mice, but inoculation with *E. coli* 1177 induced an MIP-2 response in C3H/HeN mice. MIP-2 staining was first detected 2 h after inoculation but was restricted to the pelvic epithelium and recruited neutrophil cells. At 6 h strong MIP-2 staining was detected in the pelvic epithelium, as was more diffuse staining in subepithelial cell layers (figure 4). MIP-2 staining was still detected after 24 h in both pelvic epithelium and subepithelial compartments, but the staining intensity was reduced, compared with the 6-h time point.

MIP-2 staining was weak in C3H/HeJ mice at 6 h after infection, and remained weak at 24 h (figure 4).

**Inhibition of neutrophil recruitment in C3H/HeN mice.** To examine the role of neutrophils for resistance to infection, neutrophil recruitment was inhibited in the C3H/HeN mice, and the effect on bacterial clearance was quantified. C3H/HeN mice were injected intraperitoneally with the anti-neutrophil antibody RB6-8C5 (250 μg/mouse) at 24 and 2 h prior to intra-
development of urinary tract infections between C3H/HeN and C3H/HeJ mice. Urine neutrophil numbers in C3H/HeN mice (◻) increased from background levels to reach a peak at 6 h. A neutrophil response did not occur in C3H/HeJ mice (◼). Nos. are mean ± SE, 8–10 mice per point.

Figure 2. Difference in neutrophil response to Escherichia coli urinary tract infection between C3H/HeN and C3H/HeJ mice. A. Urine neutrophil numbers in C3H/HeN mice (◻) increased from background levels to reach a peak at 6 h. A neutrophil response did not occur in C3H/HeJ mice (◼). Nos. are mean ± SE, 8–10 mice per point.

Antibody treatment of C3H/HeJ mice caused only a slight reduction in urine neutrophil counts, compared with untreated mice or those receiving control antibody (figure 5). Tissue neutrophils were not detected in these mice.

Tissue MPO measurements confirmed the difference in kidney neutrophil content between mice treated with the RB6-8C5 antibody. The RB6-8C5–treated group had lower MPO levels at 6 h (0.005 ± 0.0020 MPO units) and 24 h (0.090 ± 0.0015 MPO units) than control mice (0.024 ± 0.0040 and 0.018 ± 0.0025 MPO units, P < .05).

Neutrophil depletion impairs bacterial clearance from the urinary tract of C3H/HeN mice. The effect of neutrophil depletion on bacterial clearance from kidneys and bladders was examined in C3H/HeN mice (table 1). RB6-8C5–treated mice had 1000-fold higher bacterial counts after 24 h in the kidneys and 100-fold higher counts in the bladders than the control mice. As in the C3H/HeJ mice, the bacterial numbers in neutrophil-depleted C3H/HeN mice remained stationary or increased between 6 and 24 h. Mice treated with control antibody showed the expected reduction in bacterial counts during the same time interval.

Discussion

This study analyzed neutrophil leukocytes as antibacterial effector cells in the urinary tract. Two independent lines of evidence showed that neutrophils are essential for bacterial clearance. First, mice with inherited defects in neutrophil recruitment were unable to eliminate E. coli 1177 from kidneys and bladders. C3H/HeJ mice had a defective neutrophil response to E. coli infection, with poor clearance of bacteria from the urinary tract. Second, there was a dramatic drop in bacterial clearance from the urinary tract of normal mice after treatment with MAbs that specifically block neutrophil recruitment. Thus, in both cases the failure to clear infection was associated with defective neutrophil function. This showed that neutrophils are a major limiting factor for bacterial growth in the urinary tract during the first 24 h after infection. In the absence of neutrophils, other antibacterial defense mechanisms were unable to kill the bacterial inoculum or to limit its growth.

Neutrophil recruitment to the urinary tract involves several steps. The cells leave the circulation and migrate through the tissues to the epithelial barrier, which they then cross to reach the urinary tract lumen. In this study, the neutrophil response to UTI was characterized by detailed histology and immunohistochemistry and by quantitative urine counts. Neutrophils in tissue sections from infected animals were stained for histology with hematoxylin-eosin, and for immunohistochemistry by using the monoclonal RB6-8C5 antibodies. Neutrophils were shown to accumulate after 6 h in the kidneys of C3H/HeN mice and were still seen after 24 h (figure 3). At these time points, neutrophils were virtually absent from kidney and bladder tissues of infected C3H/HeJ mice. The results confirmed the presence of neutrophils along the mucosal barrier of resistant mice, where they are needed for the antimicrobial defense.

Table 1. Bacterial survival in kidneys and bladders after intravesical inoculation with Escherichia coli 1177.

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<tr>
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<th>C3H/HeN</th>
<th>C3H/HeJ</th>
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<tr>
<td></td>
<td>Control Ab</td>
<td>RB6-8C5</td>
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<tr>
<td>Bladders</td>
<td>3 × 10^8</td>
<td>1.1 × 10^7</td>
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<tr>
<td>Kidneys</td>
<td>1.5 × 10^7</td>
<td>3.9 × 10^6</td>
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<tr>
<td>Bladders</td>
<td>1.5 × 10^7</td>
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<tr>
<td>Kidneys</td>
<td>2.0 × 10^7</td>
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NOTE. Mice were inoculated intravesically with 1.7 × 10^8 cfa of E. coli 1177. Bacterial persistence in kidneys and bladders was determined in tissue homogenates obtained from mice killed at 6 and 24 h. Control rat IgG and RB6-8C5 were injected intraperitoneally at 24 h and again 2 h before bacterial inoculations. Values in table are geometric means of 8–10 data points.

* P < .05.
Figure 2.  (Continued) B, Neutrophils in kidney sections from C3H/HeN or C3H/HeJ mice. Tissue sections were obtained at 6 h after infection and stained with hematoxylin and eosin. Magnification ×400, arrows show neutrophil cells. Neutrophil nos. in infected tissue sections were compared with uninfected tissue (not shown). a, Neutrophils were scattered in kidney tissue, adjacent to pelvic epithelium, and were seen in renal pelvis of infected C3H/HeN mice. b, Few neutrophils were detected in kidney tissue of infected C3H/HeJ mice. c, Mild neutrophil response was detected in bladder tissue of infected C3H/HeN mice. d, Neutrophils were rare in bladders of infected C3H/HeJ mice.
Figure 3. Immunofluorescence staining of mouse neutrophils in kidneys of C3H/HeN and C3H/HeJ mice at different time points after infection. Sections were stained with antineutrophil monoclonal antibody (RB6-8C5). Magnification ×100. a. Kidney sections obtained from a C3H/HeN mouse after 6 h showed strong staining and a massive neutrophil influx into the kidney. b. Kidney sections obtained from a C3H/HeN mouse after 24 h, showed that neutrophil infiltrate had decreased compared with 6 h. c. Neutrophils were not detected in kidney sections from C3H/HeJ mice at 6 h after infection. d. Very few staining cells were detected in kidney sections from C3H/HeJ mice at 24 h after infection.

and the dearth of neutrophils in infected tissues of C3H/HeJ mice.

If this interpretation is correct, it should be possible to make the resistant C3H/HeN mice susceptible to UTI by blocking neutrophil recruitment. This was achieved by using the MAb RB6-8C5, which binds to a surface antigen present on mature granulocytes. It is cytotoxic for granulocytes but does not bind cells of the monocyte-macrophage or lymphocyte lineages [19,
In earlier studies, this antibody was used to investigate the role of neutrophils for resistance to systemic infection with *Listeria monocytogenes* [19, 20], *Salmonella typhimurium* [25, 26], and *Francisella tularensis* [27]. In the present study, RB6-8C5 treatment was shown to impair neutrophil recruitment into the kidney and bladder tissue, and as a result it decreased the available pool of neutrophils that could migrate across the epithelium into the urinary tract lumen. This neutrophil depletion was also shown to impair bacterial clearance in the C3H/HeN mouse. The bacterial counts in the kidneys of neutrophil depleted C3H/HeN mice were at least 1000-fold higher than in the C3H/HeN controls and were similar to the bacterial counts...
in C3H/HeJ mice. Treatment of C3H/HeJ mice with the RB6-8C5 antibody had only a marginal effect on bacterial clearance from kidneys, suggesting that the preexisting deficiency in neutrophil recruitment could be only marginally affected. From these results, it appears that the inhibition of neutrophil recruitment rendered the C3H/HeN mouse as susceptible to UTI as the C3H/HeJ mouse.

Early studies showed that C3H/HeN and C3H/HeJ mice differ in susceptibility to gram-negative bacterial infection [6-9] but did not directly assess the mechanisms. C3H/HeN and C3H/HeJ mice derive from the same C3H/He lineage. The C3H/HeJ mice were isolated from this original stock in the early 1960s because of their extreme resistance to intraperitoneally injected LPS [28, 29], and the LPS locus was mapped to chromosome 4 [29, 30]. The LPS-response deficiency was recently shown to result from a single amino acid substitution in Tlr4 [10]. This general deficiency in the Tlr4 receptor and ceramide signaling pathway should render all cells in the mice refractory to signals induced by LPS and other agonists that use these signaling pathways [11, 12]. The susceptibility to infection of the C3H/HeN mouse might well be caused by a deficient response in many different cell populations. Nevertheless, this study shows that neutrophils are the most critical effector cells in the urinary tract. Neutrophil-depleted C3H/HeN mice became as susceptible to infection as the C3H/HeJ mice, even though other cells were LPS responsive.

IL-8 is the main chemokine involved in neutrophil migration across Escherichia coli-stimulated human uroepithelial cell layers in vitro [14, 15]. We recently identified MIP-2, one of the mouse IL-8 homologues, as an important neutrophil chemotactant in the mouse urinary tract [14]. MIP-2 is required for peripheral neutrophil migration across the epithelium into the urine but not for the more proximal recruitment from the bloodstream into the tissues [14]. After anti-MIP-2 antibody treatment, the neutrophils accumulated under the epithelial lining of the renal pelvis, and only a fraction made it into the urine. In this study, we found a marked epithelial MIP-2 response to infection in C3H/HeN mice but a weak response in the C3H/HeJ mice. This observation suggested that a deficient mucosal chemokine response to infection is the underlying cause of the neutrophil recruitment deficiency in the C3H/HeJ mice.

So, what is the relevance of these findings for the large number of individuals with increased susceptibility to urinary tract infection? Is there evidence that neutrophil deficiencies augment the susceptibility to UTI? Neutropenic patients run an increased risk of developing gram-negative septicemia, and the urinary tract is one possible route of entry for the bacteria. In a neutrophil-proficient host, the infection might have been arrested in the urinary tract and given rise to the symptoms and signs of urinary tract infection. However, whereas neutropenia dramatically impairs the local inflammatory response, we would not expect these patients to have focal symptoms of urinary tract infections. Furthermore, the pathogenesis of urinary tract infection is multifactorial. Our studies show that neutrophils are essential for bacterial clearance from the urinary tract, once it has been infected, but do not address the relative role of different host defenses that might be involved before invasion of the urinary tract. The neutrophil data also predict that there will be more subtle deficiencies in neutrophil function among patients who suffer from recurrent UTI. Preliminary observations suggest that there is variation in human chemokine receptor expression and that low receptor expression characterizes patients with recurrent UTI and pyelonephritis. Further
Figure 6. Tissue neutrophils in RB6-8C5-treated C3H/HeN mice. Kidney sections were obtained from mice pretreated with RB6-8C5 antibody and were stained with hematoxylin-eosin (a) or with the antineutrophil antibody RB6-8C5 (b). Magnification ×100. Hematoxylin and eosin staining of a kidney section from an RB6-8C5-treated mouse. Neutrophils were absent from kidneys of these mice.

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