Evaluation of tumour necrosis factor-alpha and interleukin-1beta in an experimental pyelonephritis model induced with planktonic and biofilms cells of *Pseudomonas aeruginosa*

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Urinary tract infections may induce severe inflammation, transient impairment in renal function and scar formation, ranging in severity from acute symptomatic pyelonephritis to chronic pyelonephritis, and have the potential to lead to renal failure and death. In the present study, the relationship between production of tumour necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), neutrophil recruitment, bacterial colonization and tissue damage was investigated using a mouse model of acute ascending pyelonephritis induced with planktonic and biofilm cells of *Pseudomonas aeruginosa*. Neutrophil influx correlated with rise in TNF-α and IL-1β, indicating an association between these cytokines and neutrophil infiltration. However, biofilm cells of *P aeruginosa* induced higher levels of TNF-α and IL-1β leading to higher neutrophil infiltration causing tissue damage, assessed in terms of malondialdehyde, lactate dehydrogenase and glutathione content, which may have contributed to bacterial persistence compared with their planktonic counterparts. The results of the present investigation suggest that exaggerated cytokine production during *P aeruginosa*-induced pyelonephritis causes tissue damage operative through neutrophil recruitment leading to bacterial persistence in host tissues. The findings of the present study may be relevant for the better understanding of disease pathophysiology and for the future developments of preventive strategies against pyelonephritis based on anti-inflammatory intervention.

**Key Words:** Biofilms; IL-1β; Neutrophil recruitment; Pseudomonas aeruginosa; Pyelonephritis; TNF-α

Acute pyelonephritis is one of the most common serious bacterial infections affecting humans throughout their lifespan. Without early diagnosis and rapid treatment, the progression of renal inflammation can result in renal scarring and permanent kidney damage. Postinfectious renal scarring is the most important complication after acute pyelonephritis, with an estimated incidence of 10% to 65% of cases (1). Renal scarring later in life may lead to the subsequent development of hypertension, proteinuria and even loss of renal function that possesses several traits that contribute to its ability to colonize and persist in acute and chronic infections. These include high resistance to antimicrobials, a plethora of virulence products and metabolic versatility (8-11). In addition, *P aeruginosa* has a

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tendency to form biofilms on the surface of urinary catheters leading to recurrent and chronic infections that result in significant morbidity and mortality (12-17). Biofilm bacteria form structured communities of cells embedded in an extracellular polymeric matrix (18-21). Biofilms are resistant to antimicrobial agents as well as to host defense mechanisms, leading to difficulty in eradication (22-28). Biofilms also provide an ideal niche for the exchange of extrachromosomal DNA responsible for antibiotic resistance, virulence factors and environmental survival capabilities at accelerated rates, making it a perfect milieu for the emergence of drug-resistant pathogens (29-31).

Uropathogenic microorganisms cause disease by cytotoxic activities and also by activating a local and systemic inflammatory response. The course and severity of the disease are determined by the efficiency of the local or systemic immunological and nonimmunological defense mechanism of the host. A wide variety of cells including lymphocytes, endothelial cells and urinary tract as well as renal epithelial cells produce cytokines in response to Gram-negative bacteria or their products (32). Cytokines are small proteins important for the orchestration of the inflammatory process. The most potent proinflammatory cytokines are tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), which are produced in response to bacterial pathogens and constitute important inflammatory cytokines in various infections such as sepsis, meningitis and pyelonephritis in children (33-36). TNF-α, a proinflammatory cytokine, has been reported to play an important role in UTIs. Increased levels of this cytokine and its receptors have been observed in urine and serum samples of patients having acute pyelonephritis (37). TNF-α also stimulates production of other proinflammatory cytokines such as IL-1β. IL-1β was originally described as a fever-producing endogenous pyrogen that appears early during the inflammatory processes, and is responsible for acute-phase reactions including fever production, increased secretion of acute-phase reactants such as CRP, reactive protein and peripheral neutrophilia (38). Higher levels of this cytokine have been observed in bronchoalveolar lavage fluid of cystic fibrosis patients compared with healthy controls (39,40), indicating importance of IL-1 in the infectious process. Thus, TNF-α and IL-1β are potent multifunctional cytokines that play a central role in inflammation and immunity. No reports are available in literature regarding production of TNF-α as well as IL-1β and their role in acute pyelonephritis caused by P aeruginosa. Because P aeruginosa is the third most common pathogen, accounting for 40% of cases of catheter-associated UTIs (41), and possesses a wide arsenal of virulence factors capable of stimulating epithelial cells to secrete cytokines (42,43,9), there is a need to understand the precise contribution of cytokines in the disease process caused by this pathogen. In the present investigation, urine and renal cytokine response to bacterial infection and their role in acute pyelonephritis was studied following induction of acute pyelonephritis in mice with planktonic and biofilms cells of P aeruginosa. TNF-α and IL-1β were chosen for investigation in this study over other urinary markers because these cytokines appear early in the immune response cascade and may play a pivotal role in deciding the ultimate outcome of an infection. In addition, the assessment of these cytokines is clinically feasible in urine and potentially useful in acute pyelonephritis due to their role in systemic inflammation.

METHODS

Organisms

A urinary isolate of P aeruginosa, PA5, employed in earlier studies (44-46), isolated from a hospitalized patient having complicated UTI and serotyped as O11 by the Laboratory of HealthCare Associated Infection, London was used. This strain produced most of the recognized virulence factors such as alginate, exotoxin A, exoenzyme S, phospholipase C, protease, elastase, pyocin, pyoverd and hemolysin. In addition, a standard strain of P aeruginosa, PAO, producing most of the above-mentioned virulence factors, was obtained from Dr Barbara H Iglewski, University of Rochester, Rochester, New York. Both strains were grown overnight in nutrient broth at 37°C and harvested by centrifugation. Cells were washed three times with phosphate-buffer saline (PBS), resuspended to a concentration of 1 x 10⁸ organisms per mL in PBS (confirmed by viable cell counting) and were used as planktonic cells for induction of infection in mice.

Generation of biofilm cells

The method of Ladd et al (47) as standardized in our laboratory by Mittal et al (44) was followed for generation of biofilms. Both PA5 and PAO strains of P aeruginosa were used to generate biofilm cells. Foley catheters (Bardia, Bard Urology, United Kingdom) were cut into 1.0 cm pieces, inoculated with 100 µL of overnight grown culture and incubated at 37°C. Every 24 h the catheter pieces were removed from each flask, resuspended three times with PBS and transferred to the new flask containing fresh medium until day 4. On day 4, the catheter pieces were rinsed three times with PBS to remove adherent bacteria and sliced longitudinally into equal halves. Cells were removed from the surface of catheter pieces by scraping the inner surface with a sterile scalpel blade. The dispersed sample was then centrifuged, and the biofilm cells were suspended in 1 mL PBS. Bacterial concentration was confirmed by absorbance at 600 nm and viable counts.

Induction of ascending pyelonephritis

For induction of ascending pyelonephritis, female LACA strain mice, six to eight weeks of age, weighing 25 ± 5 g, obtained from Central Animal House, Panjab University, Chandigarh, India, were used. Animals were kept in clean polypropylene cages under pathogen-free conditions and given food and water ad libitum. The animals’ bladders were pressed to evacuate urine before instillation. Urine was checked for preinfection bacteria by culturing on selective media. A soft intramural polyethylene catheter, nonradiopaque (outer diameter 0.61 mm, Clay Adams, USA) was inserted into the bladder through the urethral meatus, and 0.05 mL of inoculum containing 10⁸ colony forming units/mL (confirmed by absorbance at 600 nm and viable counts) was slowly injected into the bladder to avoid leakage (44,45,48,49). The catheter was kept in place for 10 min after completion of instillation and then it was withdrawn carefully. In the control group, mice were injected intrarethrally with 0.05 mL of pyrogen-free saline through the catheter. No obstruction or further manipulation of the urinary tract was performed. Eight mice were used for each strain and for each time interval separately for planktonic and biofilm cell forms. All animal experiments were carried out in two groups in triplicate. The study protocol was approved by the institutional ethical committee for animal experimentation.
**Bacteriological examination**

Animals were sacrificed at one, three, five and seven days postinfection. Renal tissue was removed aseptically and sliced into two equal halves. One half was used for bacteriological examination and the other half was used for histopathology. One half of the renal tissue was then weighed and homogenized in 1 mL of sterile PBS. This homogenized tissue was plated on cetrimide agar plates. Quantitative bacterial counts per gram of the renal tissue were calculated (44).

**Histopathological examination**

The other one-half of the renal tissue was fixed in 10% buffered formal saline and was dehydrated in ethanol gradient of 30% to 100%. Tissues were then embedded in wax, sectioned and stained with hemotoxylin and eosin (50). The medulla, cortex, calyx and subcalyx of each kidney were evaluated on a semi-quantitative scale of 0 to 4. These individual scores were then added to obtain an overall severity score, which ranged from 0 to 16. The histopathological examination was performed by a pathologist experienced in urinary tract pathology.

**Cytokine response**

TNF-α and IL-1β in urine as well as in renal tissue was quantitated by using an ELISA kit (R&D Systems, USA). Urine samples were centrifuged and the supernatant was stored at −70°C for chemokine analysis. Renal tissue was removed, immediately snap frozen and stored at −70°C. Tissues were homogenized in 3 mL of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl₂ and 1 mM MgCl₂, pH 7.4, using a tissue homogenizer. Homogenates were incubated on ice for 30 min, then centrifuged at 1500×g for 10 min; supernatants were collected and passed through a 0.45 μm filter and used for ELISA. ELISA was carried out according to the manufacturer’s instructions.

**Assessment of neutrophil response**

The number of neutrophils migrating across the mucosa into the urine was quantitated in uncentrifuged urine using a hemocytometer chamber (44). Tissue neutrophils were quantitated using a myeloperoxidase (MPO) assay (40). Renal tissue was homogenized in 2 ml of 50 mM potassium phosphate, pH 6.0 with 5% hexadecyltrimethylammonium bromide and 5 mM EDTA. Homogenates were sonicated and centrifuged. Supernatants were mixed at the ratio of 1:15 with assay buffer and read at 490 nm. MPO units were calculated as the change in absorbance over time.

**Evaluation of TNF-α and IL-1β in pyelonephritis**

The virulence potential of planktonic and biofilm cells of *P. aeruginosa* was studied in vivo in a mouse model of ascending pyelonephritis. At different stages/course of infection, the presence of organisms in urine and renal tissue was checked. Peak renal bacterial counts were observed on postinfection day 1 in urine and renal tissue and peaked at postinfection day 3 in planktonic cell-infected mice and postinfection day 5 in biofilm cell-instilled mice. TNF-α and IL-1β levels were significantly higher in mice infected with biofilm cells compared with planktonic cell-instilled mice (P<0.001) (Figure 1).

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Neutrophil recruitment into renal tissue was quantitated by MPO assay. An influx of neutrophils started at postinfection day 1, reached a peak by postinfection day 3 in planktonic cell-instilled mice and on postinfection day 5 in biofilm cell-instilled mice, showing a decrease thereafter on postinfection day 7 (Figure 3). In urine, peak neutrophil infiltration was observed on postinfection day 3 in both planktonic and biofilm cell-infected mice (Figure 3). Neutrophil recruitment was significantly higher in biofilm cell-infected mice compared with planktonic cell-instilled mice (P <0.001).

**RESULTS**

In the present study, production of TNF-α and IL-1β was studied following intravesical instillation of planktonic and biofilms cells of *P. aeruginosa*. TNF-α and IL-1β was detectable at postinfection day 1 in urine and renal tissue and peaked at postinfection day 3 in planktonic cell-infected mice and postinfection day 5 in biofilm cell-instilled mice. TNF-α and IL-1β levels were significantly higher in mice infected with biofilm cells compared with planktonic cell-instilled mice (P<0.001) (Figure 1).

**Statistical analysis**

For statistical analysis of data, ANOVA, Fischer test and Student's t test was applied, and P values were calculated. P<0.05 was considered statistically significant.
the other hand, moderate inflammation was observed in renal tissue of planktonic cell-instilled mice (Figure 5C). Neutrophil infiltration was more in the renal pelvis, which extended into the medulla and cortex. Polymorphonuclear neutrophils (PMNs) were seen disseminated throughout the renal tissues in clusters in biofilm cell-instilled mice. Renal severity scores ranged from +3 to +8 in
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DISCUSSION

UTIs activate both mucosal and systemic inflammatory responses (53). Local response includes the influx of granulocytes, macrophages and monocytes as well as the production of different cytokines (54). The frequency of cytokine release as well as the amount and type of cytokines secreted from epithelial cells after bacterial attachment may determine the extent and severity of infectious process (32). Chemotactic cytokines such as TNF-α and IL-1β play a key role in...
transuroepithelial migration of polymorphonuclear cells induced by bacteria (38, 53). In the present report, we have investigated the production of TNF-α and IL-1β in a murine model of unobstructed, ascending pyelonephritis that closely mimicks human clinical situations, induced with planktonic and biofilm cells of *P. aeruginosa*. Peak production of TNF-α and IL-1β as well as bacterial counts in urine and renal tissue was observed on postinfection day 3 in mice infected with planktonic cells and on postinfection day 5 in biofilm cell-instilled experimental animals. In addition, neutrophil influx correlated with the rise in TNF-α and IL-1β at different time intervals tested, which indicates an association between these cytokines and recruitment of PMNs. However, biofilm cells of *P. aeruginosa* produced higher levels of these cytokines compared with their planktonic counterparts. In this context, Tugtepe et al (52) also observed higher levels of TNF-α in the serum of pyelonephritic rats following infection with *Escherichia coli* compared with uninfected control mice, as observed in the present study. Kinetics of IL-1 production was studied by Rudner et al (55) in *P. aeruginosa*-induced corneal infection. These workers observed peak expression of IL-1 between the first and third day, which returned to baseline preinfection levels on postinfection day 7. It was suggested that prolonged elevation of IL-1 expression contributed to corneal destruction through continued influx of neutrophils in response to increased production of macrophage inflammatory protein-2, whereas timely downregulation of IL-1 helped in bacterial clearance. It is also well known that IL-1β induces chemokines and may be involved in the scarring process partly by inducing transforming growth factor-beta and matrix metalloproteinases. It thus appears that timely downregulation of TNF-α and IL-1β may help in the bacterial clearance but require delicate balance between protective and destructive responses operative through these cytokines.

Acute pyelonephritis is accompanied with inflammatory responses leading to PMN recruitment at the site of infection (52). Neutrophils, which leave the bloodstream, migrate to the tissue, crossing the epithelial barrier and finally reaching into the lumen. In this context, therefore, study of presence of neutrophils in the urine and renal tissue carries importance. MPO assay as well as histopathology revealed neutrophil recruitment in renal tissue of mice following infection with *P. aeruginosa*. However, concomitant with the increased infiltration of neutrophils, biofilm cells of *P. aeruginosa* were still demonstrable in high numbers in urine and renal tissue of mice compared with planktonic cells, indicating that biofilm cells are resistant to neutrophil-mediated killing. In this regard, Jensen et al (56) and Takeoka et al (57) observed a lesser degree of oxidative burst activity of PMNs following stimulation by biofilm cells compared with stimulation caused by planktonic cells. Walker et al (58) showed that components

**Table 1**

|                       | Day 1 | Day 3 | Day 5 | Day 7 |
|-----------------------|-------|-------|-------|-------|
| **Severity scores**   |       |       |       |       |
| Planktonic cells      | 2±0.19| 2±0.16| 3±0.22| 3±0.25|
| Biofilm cells         | 5±0.29| 3±0.21| 6±0.34| 5±0.30|

*P<0.001 planktonic versus biofilm cells at all time intervals. PAS Urinary isolate of *Pseudomonas aeruginosa*; PAO Standard strain of *P. aeruginosa*.

![Figure 5](image_url) Histopathological examination of renal tissue of mice infected with *Pseudomonas aeruginosa*. **A** Renal tissue of saline-instilled mice (controls). **B** Severe inflammation (white arrows) along with vascular permeability (yellow arrow) was observable in renal tissue of mice following infection with biofilm cells of *P. aeruginosa*. On the other hand, moderate inflammation (yellow arrows) was seen in renal tissue of mice infected with planktonic cells of *P. aeruginosa* (**C**).
from neutrophils could serve as a biological matrix to facilitate 
P. aeruginosa biofilm formation. These authors reported that human neutrophils could serve to enhance the initial development of P. aeruginosa biofilms.

Available experimental data from the literature confirm that products of lipid-peroxidation act as markers for determining the extent of tissue damage. To measure this, researchers have quantitated LDH and MDA levels for estimating the intensity of lipid peroxidation in tissues because it offers speed, reliability and sensitivity (52). In addition, pyelonephritis has been reported to be accompanied with oxidative stress leading to depletion in renal GSH. GSH is the major antioxidant produced by the cell, protecting it from free radicals. It plays a crucial role in maintaining a normal balance between oxidation and anti-oxidation. GSH deficiency contributes to oxidative stress and therefore appears to play a key role in the pathology of many diseases. In the present study, biofilm cells of P. aeruginosa produced increased TNF-α indicating oxidative stress and tissue damage,

findings, it was previously shown that depletion of PMNs in rats reduces the renal destruction in exudative E. coli pyelonephritis and protects from chronic parenchymal destruction and scarring (60). Moreover, it was demonstrated that permanent tissue damage, which occurs during acute pyelonephritis, could be prevented through the inhibition of MPO system (61).

CONCLUSIONS

The results of the current study suggest that TNF-α and IL-1β production may have the potential to influence the establishment of UTI. The findings of this investigation can be gainfully employed for future studies where profile of TNF-α and IL-1β induced by different uropathogens may be studied critically following infection. A better knowledge of chemokine action and regulation during pyelonephritis may therefore help to explore new therapeutic strategies in the future with far reaching consequences.

ACKNOWLEDGEMENTS: We are thankful to Judith Glover, Laboratory of HealthCare Associated Infection, London, for serotyping of Pseudomonas aeruginosa. We are also grateful to Dr Barbara H Iglewski for providing us with a standard strain of P. aeruginosa. This work was supported by a grant from Indian Council of Medical Research, New Delhi, India.

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