RESEARCH ARTICLE

Contribution of macrophage secretory products to urovirulence of Pseudomonas aeruginosa

Rahul Mittal1,2, Sudhir Aggarwal3, Saroj Sharma2, Sanjay Chhibber2 & Kusum Harjai2

1Division of Infectious Diseases, Children’s Hospital Los Angeles, Los Angeles, CA, USA; 2Department of Microbiology, Panjab University, Chandigarh, India; and 3Department of Physiology, University of Tennessee Health Sciences Center, Memphis, TN, USA

Correspondence: Rahul Mittal, Division of Infectious Diseases, MS #51, Children’s Hospital Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90027, USA. Tel.: +1 323 361 5809; fax: +1 323 361 6004; e-mail: ramittal@chla.usc.edu

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urinary tract infections; macrophage secretory products; Pseudomonas aeruginosa; biofilms.

Abstract

Macrophages form one of the first lines of defense on mucosal surfaces like urinary tract, providing protection against pathogens. These cells pour their secretory products, which include a cocktail of biomolecules, at the site of infection. In the present investigation, the effect of macrophage secretory products (MSPs) obtained after interaction of macrophages with Pseudomonas aeruginosa on the virulence of this organism in planktonic and biofilm cell mode was assessed employing a mouse model of ascending pyelonephritis. When urinary tract infection (UTI) was established with P. aeruginosa grown in the presence of 30% MSPs, the extent of pyelonephritis was enhanced. Of the two cell forms, biofilm cells had an edge over the planktonic cells with respect to in vivo virulence. The enhanced virulence of MSP-grown P. aeruginosa may be attributed to increased production of quorum-sensing systems as well as increased adherence to uroepithelial cells and evasion of phagocytosis. The results of the present study reveal that macrophages can play a key role during the course of UTI, not only through their phagocytic activity, but also through effects mediated by their secretory products. Utilization of MSPs by P. aeruginosa can have far-reaching consequences, including chronicity and recurrence of infections caused by this pathogen.

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span (Drekonja & Johnson, 2008). While trying to establish in the urinary tract, the invading organism comes in contact with phagocytes, primarily macrophages (Stuart & Ezekowitz, 2005). These cells are specialized phagocytes that play an important role in clearance of effete host cells and molecules as well as in immune defense against foreign invasion and infection (Jutras & Desjardins, 2005). Earlier, Nathan (1987) reviewed the subject and stated that macrophages of tissues as well as those coming from the circulation congregate in the most acute and chronic inflammatory reactions. Macrophages respond to antigenic stimuli and secrete a range of over 100 substances, which vary in their biological activities, affecting induction of cell growth, cell death and multiple metabolic functions. This cocktail of secretory products of macrophages includes peptide hormones, complement components, enzymes, bioactive oligopeptides, lipids, reactive oxygen species, reactive nitrogen intermediates and other biological substances (Nathan, 1987). The principal constituents of macrophage secretory products (MSPs) are cytokines like tumor necrosis factor-α (TNF-α) and interleukin-β (IL-1β).

Pseudomonas aeruginosa is the most common pathogen associated with hospital-acquired catheter-associated UTIs (Jarvis & Martone, 1992). It possesses a wide arsenal of weapons capable of causing considerable damage to the host. The virulence of P. aeruginosa has been reported to be multifactorial, associated with a number of cell-associated and cell-free virulence determinants such as alginate, flagellum, pilus and nonpilus adhesins, protease, elastase, phospholipase, pyocyanin, exotoxin A, exoenzyme S, hemolysins (rhamnolipids) and siderophores regulated by quorum-sensing systems (Visca et al., 1992; Girard & Bloemberg, 2008; Le Berre et al., 2008). Two types of quorum-sensing systems, las and rhl, have been described in P. aeruginosa (Smith & Igleswki, 2003; Juhas et al., 2005; Parker & Sperandio, 2009). These quorum-sensing systems operate through autoinducers. Acylhomoserine lactones play an important role in the pathogenesis of P. aeruginosa-induced
infections (Kumar et al., 2009; Lesic et al., 2009; Nelson et al., 2009). In addition, P. aeruginosa has a tendency to form biofilms on the surface of indwelling catheters (Hoiby et al., 2001). The biofilm mode of life conveys a survival advantage to this pathogen and thus results in persistent infections that are resistant to antimicrobial therapy and host defense mechanisms (Hatt & Rather, 2008). It is not known how this pathogen persists in the host tissues despite the presence of potent immune cells such as macrophages. As P. aeruginosa causes recurrent and chronic infections that are difficult to eradicate, there is a need to understand how this pathogen subverts the host immune system for its own growth and survival. The current study was undertaken to examine the effect of MSPs on virulence of P. aeruginosa in a mouse model of acute pyelonephritis. Here we demonstrate for the first time that MSPs augment the onset of acute pyelonephritis and play an important role in P. aeruginosa-induced UTIs.

**Materials and methods**

**Bacteria**

Two urinary isolates (PA3 and PA5) of P. aeruginosa, serotypes O6 and O11 (serotyped by the Laboratory of Health Care Associated Infection, London), isolated from hospitalized patients with complicated UTI and employed in earlier studies were used (Mittal et al., 2004, 2009a–c; Harjai et al., 2005). In addition, a standard strain of P. aeruginosa, PAO, was obtained from Dr Barbara H. Iglewski, University of Rochester, Rochester, NY. All the strains were grown overnight in RPMI medium containing 30% MSPs collected from unstimulated macrophages, which served as control and macropages stimulated with P. aeruginosa at 37 °C under shaking conditions and harvested by centrifugation. Cells were washed three times with phosphate-buffered saline (PBS), resuspended to a concentration of 1 × 10^8 organisms mL^-1 in PBS (confirmed by viable counts) and used as planktonic cells for induction of infection in mice.

**Generation of biofilm cells**

For generation of biofilms, Foley’s catheter (Bardia™) was cut into 1.0-cm pieces and put in flasks containing RPMI-1640 medium with 30% MSPs collected from unstimulated (control) and stimulated macrophages (Ladd et al., 1987; Mittal et al., 2004, 2008b, 2009a–c; Harjai et al., 2005). These were inoculated with 100 μL of overnight culture and incubated at 37 °C. Every 24 h, catheter pieces were removed from each flask, rinsed three times with PBS (pH 7.2) and transferred to a new flask containing fresh medium until day 4. On day 4, 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, washed three times with PBS and then suspended in 1 mL PBS. Bacterial concentration was confirmed by viable counts.

**Isolation of peritoneal macrophages and preparation of MSPs**

Peritoneal macrophages were isolated from a pathogen-free LACA strain of mice using the method of Mittal et al. (2008b). Macrophages (10^6 cells mL^-1) were interacted with each bacterial strain (10^8 CFU mL^-1) at a multiplicity of infection of 1:100 separately in planktonic and biofilm cell mode. These were kept at 37 °C for 20 min. This time period allowed optimal phagocytosis of bacteria by macrophages. The macrophages were then repeatedly washed with RPMI-1640 to remove any unphagocytosed adherent bacteria. After resuspending in serum and antibiotic-free RPMI-1640, the macrophages were kept at 37 °C in a 5% CO2 environment. Supernatant (MSPs) was collected at 18-h postinteraction time period (Mattana & Singhal, 1993; Sharma et al., 1996). This was then passed through 0.2-μm filter and stored at −80 °C before experiment. Sterility of MSPs was checked before setting up each experiment by plating it on nutrient agar plates. Supernatants collected from unstimulated macrophages served as control to rule out nonspecific stimulation of macrophages during handling procedures.

**Characterization of MSPs**

MSPs were characterized in terms of cytokines, reactive nitrogen intermediates (both nitrates and nitrites) and protein content. Cytokines, namely TNF-α, TNF-β, IL-1α, IL-1β, IL-2, granulocyte monocyte colony stimulating factor (GM-CSF), macrophage-inflammatory protein-2 (MIP-2), IL-6, IL-10, IL-12 and IL-18 were measured using ELISA kits (R&D, Minneapolis, MN). Reactive nitrogen intermediates were measured using the method of Rockett et al. (1994).

**Phagocytosis assay**

A total of 0.4 mL of normal mouse serum, 0.5 mL of macrophage cell suspension (2 × 10^6 cells mL^-1) and 0.1 mL of bacterial suspension (10^8 cells mL^-1), were placed in separate duplicate test tubes, vortexed gently and incubated at 37 °C under 5% CO2 atmosphere. Aliquots were taken after 90 min of incubation and were transferred to 2 mL of cold RPMI-1640 medium. Macrophages were pelleted by centrifugation, and the viable count of bacteria in the supernatant was determined by plating appropriate serial dilutions on nutrient agar plates. Results were expressed as percentage bacteria taken up by the macrophages by
subtracting the count in supernatant from the total count added.

**Uroepithelial cell (UEC) adhesion assay**

UEC adhesion assay was performed following the method of Mittal et al. (2008b). Urine samples from healthy females in the age group between 20 and 25 years were screened for regular shedding of UECs. Early morning pooled urine samples from four healthy females (300 mL from each subject) who were regular shedders were used for the collection of UECs. Only the females who were not taking any antibiotics or other supplements were included in this study. Throughout the study period, urine was collected from these volunteers. Urine was centrifuged at 1500 g for 15 min at 4 °C and sediment containing UECs was suspended in PBS. UECs were washed three times with PBS, pH 7.0. A drop of trypan blue dye was added to the final suspension to identify dead UECs. The cell count of the viable cells was adjusted to 10^5 cells mL^-1 using a haemocytometer. An adhesion assay mixture consisting of 1 mL each of bacterial cells and UECs was incubated at 37 °C for 1 h. After centrifugation and a minimum of three washings to remove unattached bacteria, smears were prepared and stained with Giemsa stain. Bacteria adhering to 30 UECs were counted, and the average number of bacteria adhering per UEC was calculated.

**Determination of quorum-sensing systems**

*Escherichia coli* MG4 (pKDT17) was used as reporter strain for detection of acyl homoserine lactones, kindly provided by Dr Barbara H. Iglewski, University of Rochester, NY. Plasmid pKDT17 contains LasR under control of the lac promoter and a LasB::LacZ translational fusion. The details of the strain have been given earlier (Pesci et al., 1999). The culture supernatants of planktonic and biofilm cells grown in the absence and presence of 30% MSPs were extracted twice with ethyl acetate containing 0.01% acetic acid (Zhu et al., 2002). Plasmid pKDT17 contains LasR under control of the lac promoter and a LasB::LacZ translational fusion. The details of the strain have been given earlier (Pesci et al., 1999). The culture supernatants of planktonic and biofilm cells grown in the absence and presence of 30% MSPs were extracted twice with ethyl acetate containing 0.01% acetic acid (Zhu et al., 2002). Reporter strain 2 mL and extracted supernatant 0.5 mL was incubated at 30 °C in a water bath for 5 h with rotation at 100 r.p.m. Culture was centrifuged at 4500 g for 15 min. Pellets were suspended in equal volumes of Z buffer [Na2HPO4·7H2O (0.06 M), NaH2PO4·H2O (0.04 M), KCl (0.01 M), MgSO4·7H2O (0.001 M), β-mercaptoethanol (0.05 M), pH 7.0]. To 1 mL of cell culture, 1 mL of Z buffer, 200 μL chloroform and 100 μL of 0.1% SDS were added to lyse cells. O-nitrophenol-β-D-galactopyranoside (0.4 mL) (4 mg mL^-1 in PBS) was added. Reaction was stopped by adding 1 mL of 1M Na2CO3 after the development of yellow color. OD was taken at 420 nm. Units of β-galactosidase were then calculated according to the method of Zhu et al. (2002).

**Induction of ascending pyelonephritis**

Pathogen-free female LACA mice, 6–8 weeks old, weighing 25 ± 5 g were obtained from Central Animal House, Panjab University, Chandigarh, India. A soft intramedic polyethylene catheter, nonradioopaque (outer diameter 0.61 mm; Clay Adams, Parsippany, NJ) was inserted into the bladder through the urethral meatus, and 0.05 mL of inoculum containing 10^8 CFU mL^-1 was slowly injected into the bladder to induce leakage (Hagberg et al., 1983; Mittal et al., 2004, 2008a, b; Harjai et al., 2005). The catheter was kept in place for 10 min after completion of instillation and then it was withdrawn carefully. No obstruction or further manipulation of the urinary tract was done.

To study the effect of MSPs on *in vivo* colonization, planktonic and biofilm cells of *P. aeruginosa* grown in 30% MSPs were used to induce acute ascending UTI. 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 1-, 3-, 5- and 7-day postinfection. One-half of both the kidneys and the bladder tissue was removed aseptically, 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 kidney and bladder tissue were calculated (Mittal et al., 2004).

**Histopathological examination**

The other half of the kidney and bladder tissue was fixed in 10% buffered formal saline and was dehydrated in ethanol gradient of 30–100%. Tissues were then embedded in wax, sectioned and stained with haematoxylin and eosin (Garg et al., 1987). The medulla, cortex, calyx and subcalky of each kidney were evaluated on a semi-quantitative scale of 0–4. These individual scores were then added to obtain an overall severity score, which ranged from 0 to 16. Bladder tissue was scored on a semi-quantitative scale of 0–4 according to the method of Hopkins et al. (1998).

**Malondialdehyde estimation**

Malondialdehyde was estimated following the method of Mittal et al. (2004). Briefly, tissue supernatant or urine was added to an equal amount of Tris-HCl (0.1 M, pH 7.4) and incubated at 37 °C for 2 h. After incubation, trichloroacetic acid was added and centrifuged at 700 g for 10 min. Supernatant was mixed with an equal volume of thiobarbituric acid (0.67% w/v) and kept in boiling water bath for 10 min.
After cooling, the volume was made up to 3 mL with double distilled water and absorbance was taken at 532 nm. The amount of malondialdehyde formed was expressed in nmol mg⁻¹ protein using an extinction coefficient of 1.56 × 10³ M⁻¹ cm⁻¹.

Statistical analysis

For statistical analysis of data, ANOVA, Fisher’s test and Student’s t-test were applied, and P values were calculated. P < 0.05 was considered statistically significant.

Results and discussion

The innate immunity provides a first line of defense against pathogens in which macrophages play an important role. Macrophages, coming mostly from the circulation, form one of the initial lines of defense in the urinary tract and offer resistance against infection (Stuart & Ezekowitz, 2005). These macrophages interact with invading pathogens, leading to elaboration of biochemical substances referred to as MSPs. In earlier studies it has been reported that a 30% concentration of MSPs modulates host cell responses (Mattana & Singhal, 1993; Sharma et al., 1996); this concentration of MSPs has therefore been employed in the present investigation. Further, employing secretory products of macrophages more closely simulates the in vivo environment of host-parasite interaction. Characterization of MSPs revealed that they contain a variety of cytokines such as TNF-α, TNF-β, IL-1α, IL-1β, GM-CSF, MIP-2, IL-6, IL-12 and IL-18, as well as reactive nitrogen intermediates (data not shown). Conversely, none of the cytokines was detectable in supernatants collected from unstimulated macrophages. In the present study, when UTI was established in mice with planktonic and biofilm cells of *P. aeruginosa* grown in medium containing 30% MSPs, it was observed that the organisms were significantly more virulent than untreated bacteria, as indicated by increased bacterial load (Table 1), malondialdehyde production (a marker of tissue damage) and cytokine secretion (Table 1).

### Table 1a. Bacterial load in mice following infection with planktonic cells of uroisolates (PA3 and PA5) and standard strain (PAO) of *Pseudomonas aeruginosa* grown in the presence of 30% MSPs

<table>
<thead>
<tr>
<th>Strains</th>
<th>Tissue</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA3</td>
<td>Kidney</td>
<td>4.32 ± 0.42</td>
<td>5.79 ± 0.32</td>
<td>6.89 ± 0.40</td>
<td>7.28 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2.45 ± 0.32</td>
<td>3.84 ± 0.32</td>
<td>4.43 ± 0.40</td>
<td>4.92 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>4.31 ± 0.32</td>
<td>6.43 ± 0.32</td>
<td>8.20 ± 0.40</td>
<td>8.06 ± 0.43</td>
</tr>
<tr>
<td>PA5</td>
<td>Kidney</td>
<td>4.43 ± 0.42</td>
<td>5.92 ± 0.35</td>
<td>6.95 ± 0.40</td>
<td>7.39 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2.56 ± 0.35</td>
<td>4.94 ± 0.35</td>
<td>5.48 ± 0.40</td>
<td>5.98 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>4.21 ± 0.35</td>
<td>6.81 ± 0.35</td>
<td>8.15 ± 0.40</td>
<td>8.05 ± 0.43</td>
</tr>
<tr>
<td>PAO</td>
<td>Kidney</td>
<td>4.32 ± 0.40</td>
<td>5.85 ± 0.36</td>
<td>6.85 ± 0.40</td>
<td>7.33 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2.49 ± 0.36</td>
<td>4.89 ± 0.36</td>
<td>4.49 ± 0.40</td>
<td>4.94 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>4.28 ± 0.36</td>
<td>6.88 ± 0.36</td>
<td>8.17 ± 0.40</td>
<td>7.98 ± 0.42</td>
</tr>
</tbody>
</table>

*P < 0.01.
**P < 0.001, control vs. test.

### Table 1b. Bacterial load in mice following infection with biofilm cells of *Pseudomonas aeruginosa* grown in the presence of 30% MSPs

<table>
<thead>
<tr>
<th>Strains</th>
<th>Tissue</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA3</td>
<td>Kidney</td>
<td>5.51 ± 0.52</td>
<td>6.82 ± 0.47</td>
<td>7.92 ± 0.55</td>
<td>8.52 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3.53 ± 0.47</td>
<td>5.94 ± 0.47</td>
<td>5.52 ± 0.55</td>
<td>4.96 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>5.35 ± 0.47</td>
<td>7.52 ± 0.47</td>
<td>9.18 ± 0.55</td>
<td>9.51 ± 0.49</td>
</tr>
<tr>
<td>PA5</td>
<td>Kidney</td>
<td>5.62 ± 0.54</td>
<td>6.97 ± 0.48</td>
<td>7.98 ± 0.53</td>
<td>8.67 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3.67 ± 0.48</td>
<td>6.45 ± 0.48</td>
<td>6.33 ± 0.53</td>
<td>5.15 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>5.43 ± 0.48</td>
<td>7.85 ± 0.48</td>
<td>9.29 ± 0.53</td>
<td>9.50 ± 0.51</td>
</tr>
<tr>
<td>PAO</td>
<td>Kidney</td>
<td>5.58 ± 0.54</td>
<td>6.89 ± 0.50</td>
<td>7.95 ± 0.50</td>
<td>8.51 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3.61 ± 0.50</td>
<td>6.24 ± 0.50</td>
<td>5.57 ± 0.50</td>
<td>5.03 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>5.40 ± 0.50</td>
<td>7.92 ± 0.50</td>
<td>9.10 ± 0.50</td>
<td>9.22 ± 0.58</td>
</tr>
</tbody>
</table>

*P < 0.01.
**P < 0.001, control vs. test.
damage) (Fig. 1), and tissue pathology assessed in terms of severity scores (Table 2). In addition, a significant increase in the production of quorum-sensing systems was observed following the growth of organisms in 30% MSPs compared with untreated bacteria ($P < 0.001$) (Fig. 2). This effect of MSPs was more pronounced in the case of biofilms cells compared with planktonic cells of $P. aeruginosa$. Histopathological examination of renal tissue of mice infected with $P. aeruginosa$ cultured in the presence of MSPs (test group) showed marked inflammation along with abscess, casts, obliteration of tubules, shedding of cells and vascular permeability (Fig. 3a) compared with organisms grown in the presence of MSPs from unstimulated macrophages or in the absence of MSPs (control group), where only moderate inflammation was observable (Fig. 3b). Bladder tissue also showed severe inflammation along with shedding of cells and vascular permeability, leading to loss of normal architecture of tissue in the test group (Fig. 3c). In contrast, moderate inflammation was seen in bladder tissue of the control group (Fig. 3d). Because of the paucity of literature, no direct comparisons are possible. However, interaction of $E. coli$ with macrophages has been reported to lead to proliferation of mesangial cells and matrix synthesis in vitro in a renal tissue cell culture model (Sharma et al., 1996). Mesangial cell expansion is a precursor for focal glomerulosclerosis, and hence, MSPs produced by bacterial interaction contributed to this expansion, which is a characteristic feature of pyelonephritis. The increased virulence of the organism observed in the present study may be due to the enhanced development of glomerular lesions in renal tissue, which may form a niche for the growth and multiplication of the organism. In addition, cytokines, which are principal constituents of MSPs, have been reported to influence growth and virulence of pathogens both in vitro and in vivo. Luo et al. (1993) reported enhanced invasion of HeLa cells in vitro by Shigella flexneri following binding of TNF-α on the surface of this pathogen. The binding of cytokines, including TNF-α, to the bacterial cells has also been studied by Meduri et al. (1999), who reported that these molecules are internalized, and bacteria break cytokines down to
Interaction of P. aeruginosa with MSPs

Table 2a. Kidney and bladder severity scores of mice following infection with planktonic cells of Pseudomonas aeruginosa grown in the presence of 30% MSPs

<table>
<thead>
<tr>
<th>Strains</th>
<th>Tissue</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA3</td>
<td>Kidney</td>
<td>2 ± 0.22</td>
<td>5 ± 0.45</td>
<td>6 ± 0.26</td>
<td>6 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2 ± 0.19</td>
<td>3 ± 0.38</td>
<td>3 ± 0.15</td>
<td>3 ± 0.28</td>
</tr>
<tr>
<td>PA5</td>
<td>Kidney</td>
<td>2 ± 0.11</td>
<td>5 ± 0.19</td>
<td>6 ± 0.24</td>
<td>6 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2 ± 0.26</td>
<td>3 ± 0.34</td>
<td>2 ± 0.31</td>
<td>3 ± 0.28</td>
</tr>
<tr>
<td>PAO</td>
<td>Kidney</td>
<td>2 ± 0.14</td>
<td>3 ± 0.15</td>
<td>6 ± 0.22</td>
<td>8 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>2 ± 0.25</td>
<td>2 ± 0.24</td>
<td>1 ± 0.11</td>
<td>2 ± 0.16</td>
</tr>
</tbody>
</table>

*P < 0.01.
**P < 0.001, control vs. test.

Table 2b. Kidney and bladder severity scores of mice following infection with biofilm cells of Pseudomonas aeruginosa grown in the presence of 30% MSPs

<table>
<thead>
<tr>
<th>Strains</th>
<th>Tissue</th>
<th>1 Day</th>
<th>3 Days</th>
<th>5 Days</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA3</td>
<td>Kidney</td>
<td>4 ± 0.38</td>
<td>7 ± 0.72</td>
<td>5 ± 0.45</td>
<td>8 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3 ± 0.29</td>
<td>4 ± 0.48</td>
<td>2 ± 0.57</td>
<td>4 ± 0.34</td>
</tr>
<tr>
<td>PA5</td>
<td>Kidney</td>
<td>5 ± 0.58</td>
<td>8 ± 0.78</td>
<td>6 ± 0.34</td>
<td>8 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3 ± 0.47</td>
<td>4 ± 0.67</td>
<td>3 ± 0.61</td>
<td>4 ± 0.38</td>
</tr>
<tr>
<td>PAO</td>
<td>Kidney</td>
<td>3 ± 0.38</td>
<td>7 ± 0.59</td>
<td>5 ± 0.49</td>
<td>7 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>3 ± 0.61</td>
<td>3 ± 0.25</td>
<td>2 ± 0.14</td>
<td>3 ± 0.24</td>
</tr>
</tbody>
</table>

*P < 0.01.
**P < 0.001, control vs. test.

Fig. 2. Effect of MSPs on quorum-sensing systems. Planktonic (a) and biofilm cells (b) of Pseudomonas aeruginosa were generated in the absence (control) and presence (test) of 30% MSPs and production of quorum-sensing systems was assessed as described in Materials and methods. The data represent mean ± SD values and are representative of six independent experiments carried out in triplicate. *P < 0.001 control vs. test.

biologically active fragments with a capacity to be transported across the bacterial cell membrane. These then act on transcription and translation of specific genes, leading to alteration in the virulence properties of the organism. In relation to pneumonia, another example of mucosal infections, Lee et al. (2003) by employing TNF-α knockout mice reported that the host’s response plays a more decisive role than bacterial virulence factors in the elimination and clearance of the invading pathogen. These findings are in agreement with the results of the present study, where we observed that P. aeruginosa utilizes MSPs to increase the expression of quorum-sensing systems, resulting in enhanced virulence in vivo.

The pathogenic microorganisms must adhere to host cell surfaces to gain a foothold for further interactions that result in establishment and disease (Finlay & Falkow, 1997). In this context, in relation to UTIs, the capacity of the microorganism to adhere to UECs is crucial in the initial colonization of the bladder mucosa (Mulvey, 2002). Bacteria unable to adhere remain suspended in the urine and are removed by voiding. Eden et al. (1976, 1981) have shown that E. coli isolates obtained from patients with cystitis adhere to UECs in significantly higher numbers than do E. coli fecal isolates. In the present study, we observed significant enhancement in adherence of P. aeruginosa to UECs following growth in MSPs (Fig. 4a). The average
number of bacteria per UEC in planktonic and biofilms cells of *P. aeruginosa* respectively varied from 41 to 50 and from 72 to 87 in the presence of MSPs, whereas it ranged from 19 to 27 and 35 to 44 in absence of MSPs. In the presence of a larger inoculum of bacteria binding to UECs, especially with good adhesive qualities, the primary defense of antiadherence may be overcome, colonization can occur (which is no longer transient) and bladder infection may result. Interestingly, *P. aeruginosa* was also able to evade phagocytosis after being coated with these secretory products (Fig. 4b). This increased adhesion to UECs and evasion of phagocytosis as well as increased elaboration of virulence factors by *P. aeruginosa* grown in MSPs, as observed in earlier studies from our laboratory (Mittal *et al.*, 2006), may account for its enhanced virulence observed in vivo.

In summary, our studies have directly implicated MSPs as capable of enhancing the extent of pyelonephritis, thus supporting that macrophages play a pivotal role in UTIs. The results of the present study suggest that *P. aeruginosa* can utilize macrophages and their secretory products for its...
growth and survival, leading to persistent and recurrent infections. Further studies are warranted, especially in vivo, to understand the pathophysiology of pyelonephritis in relation to the specific role of host macrophages and their secretory products.

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References


