Urovirulence of *Pseudomonas aeruginosa*: Planktonic Cells vs. Biofilm Cells

V. Yadav\(^a\), K. Harjai\(^a\), R. Kaur\(^a\), K. Joshi\(^b\), S. Sharma\(^a*\)

\(^a\)Department of Microbiology, Panjab University and \(^b\)Department of Pathology, Post Graduate Institute of Medical Education and Research, Chandigarh 160 014, India

Fax 0172 541 409

Received 25 September 2003
Revised version 16 March 2004

ABSTRACT. Planktonic and biofilm cells of a clinical urinary isolate of *P. aeruginosa* were compared *in vitro* for their ability to adhere to uroepithelial cells, interaction with macrophages, and for production of virulence factors like extracellular proteinase, elastase, hemolysin, phospholipase C and pyochelin. Biofilm cells showed increased adherence to UECs, which was coupled with reduced uptake and intracellular killing by macrophages. Overall there was a decrease in production of extracellular products by biofilm cells. Comparing the two cell forms for their ability to establish infection in an ascending model of acute pyelonephritis, significant enhancement of renal bacterial load, as well as more pronounced renal pathology developed with biofilm cells.

Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>UEC</td>
<td>Uroepithelial cell</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
</tbody>
</table>

*P. aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues. If tissue defenses are compromised in some manner, there is hardly any tissue of the body that it cannot infect. It is the third leading cause of hospital-acquired UTIs, accounting for about 11% of nosocomial UTIs (CDC 1983). UTIs caused by *P. aeruginosa* are usually associated with catheterization, instrumentation or surgery (Leigh and Emmanuel 1984). It has a tendency to colonize catheter surfaces forming a biofilm which have been demonstrated *in vitro*, as well as *in vivo* on medical devices (Hoiby et al. 2001; Kadry 2003). Despite the fact that biofilm cells of *P. aeruginosa* can pose a life-threatening problem, especially in patients dependent on medical devices, pathogenesis of catheter-associated UTIs caused by biofilm cells of *P. aeruginosa* has not been well studied.

Here we have compared biofilm cells of *P. aeruginosa* with planktonic cells for their ability to adhere to UECs, for uptake by macrophages, and for elaboration of extracellular virulence factors *in vitro*, keeping in mind the initiation and establishment of infection *in vivo*. Further, to understand their importance *in vivo*, the course of establishment of acute ascending pyelonephritis has been compared in normal mice with biofilm cells and planktonic cells of *P. aeruginosa*.

MATERIAL AND METHODS

* Bacterial isolates. *P. aeruginosa* uroisolate PU7 (Yadav et al. 2000) exhibiting extracellular virulence factors (pyochelin, proteinase, elastase, PLC, hemolysin) and adherence to UECs was used. The isolate was maintained on nutrient agar slants.

* Planktonic cell preparation. Single isolated bacterial colony obtained on nutrient agar plate was transferred to 100 mL trypticase soya broth and incubated for 18 h at 37 °C. Cells were harvested by centrifugation (2350 g, 15 min). Bacterial pellets so obtained were given 3 washings with PBS (pH 7.2) and the counts were adjusted to the desired concentration.

* Biofilm formation. Biofilm was established on Foley’s catheter according to Shigeta et al. (1997). Foley’s catheter (Bardia\(^TM\)) was cut into 10-mm pieces, put in trypticase soya broth and inoculated with overnight culture. Catheter pieces were transferred to fresh medium after every 1 d of incubation at 37 °C.

*Corresponding author.
After 4 d, the number of biofilm cells adhering to the catheter were determined according to Ababio et al. (1999). Catheter pieces were removed, rinsed thrice with PBS and sliced longitudinally into two halves. Cells were removed from the catheter surface pieces by scraping the inner surface with a scalpel blade. Two low-level sonication cycles of 30 s each were given followed by vortexing for 30 s. Dispersed sample was then centrifuged and pellet was washed and suspended in PBS to the desired concentration.

**Animals.** Female BALB/c mice procured from Central Animal House, Panjab University, Chandigarh (India), weighing 20–30 g were fed on standard antibiotic-free synthetic feed (JDB Agencies Private, India).

**Extracellular enzyme production.** Elastolytic activity was determined according to Ohman et al. (1980). For basic proteolytic activity the method of Howe and Iglewski (1984) was followed. PLC production was observed on egg yolk-agar plates (Haberman and Hardet 1972). In each case, culture supernatants of planktonic and biofilm cells were added to the wells in the plates, and zone of clearance around the well was observed. For qualitative assessment of hemolysin activity, zones of clearance around bacterial colonies of planktonic and biofilm cell forms on 5 % sheep blood agar plates were observed.

**Pyochelin production** was studied according to Arnow (1937). Cultures of planktonic and biofilm cells were grown in M9 medium with and without Pyochelin was estimated in the supernatant and absorbance at 510 nm was read.

**Phagocytosis.** Uptake and intracellular killing of *P. aeruginosa*, planktonic cells and biofilm cells by mouse peritoneal macrophages was studied according to Allen et al. (1987) method with slight modifications. Macrophage suspension was mixed with diluted mouse serum, bacterial suspension (2 × 10⁸ CFU/mL) and incubated at 37 °C in 5 % CO₂ atmosphere. At intervals, 20-μL aliquots of the mixture were suspended in cold RPMI-1640 medium for uptake assay. Macrophages were pelleted and the number of viable bacteria in the supernatant was determined by plating appropriate serial dilutions in triplicate on nutrient agar plates. Relative uptake value was expressed as percentage of viable bacteria from the initial inoculum remaining in the mixtures at each sampling time. For intracellular killing the bacterial suspension was mixed with normal mouse serum and kept for 30 min at room temperature. This was washed with normal saline and added to macrophages suspended in endotoxin-free RPMI-1640 supplemented with 5 % FCS. This suspension was incubated for 20 min at 37 °C to allow phagocytosis. The free bacteria were removed by centrifugation and subsequent washings with PBS (0.2 mol/L, pH 7.2). Finally, the pellet was suspended in 2 mL RPMI-1640 containing FCS and gentamicin; it was incubated at 37 °C. Macrophages were lysed at intervals, CFU being counted after overnight incubation.

**Serum bactericidal assay** was done according to Taylor (1983). Planktonic and biofilm cells of uroisolate PU7 were incubated with pooled human serum. Aliquots were plated at 0, 1, 2 and 3 h of incubation. Based on colony counts, organisms were divided into 3 groups: completely serum-resistant, promptly serum-sensitive, and delayed serum-sensitive.

**Scanning electron microscopy.** Biofilm samples were prepared for scanning electron microscopy by fixation with 2.5 % glutaraldehyde. The samples were dehydrated, air-dried, sputter-coated and visualized.

**Induction of acute pyelonephritis.** Original method of Hagberg et al. (1983) with slight modification was used for inducing acute ascending pyelonephritis; 0.1 mL of inoculum (planktonic and biofilm cells) containing 2 × 10⁸ bacteria per mL, was introduced into the bladder through a soft intramedic-noradiopaque polyethylene tubing with an outer diameter of 0.61 mm (Clay Adams, USA). Later the catheter was withdrawn carefully.

Mice infected with the planktonic and biofilm cells were sacrificed after 5, 7, 12 and 24 d p.i. Kidneys were removed aseptically, and were examined bacteriologically (Kakkar et al. 1986), and histopathologically (Garg et al. 1987). Blood cultures were done to exclude bacteremia. The experiments were carried out in triplicate.

**Statistical analysis.** Results were analyzed statistically by Student’s *t*-test. The probability (*p*) of obtaining values for a given degree of freedom was determined by comparing the ‘*t*’ value.

**RESULTS**

Biofilm was established with uroisolate on Foley’s catheter. Organisms adhering to the catheter surface following 1 d of incubation were revealed by cell count and scanning electron microscopy. An increase in the biofilm cell density was observed with time. Five days after incubation, the catheter surface was covered with bacterial biofilm composed of bacteria enmeshed in extracellular slimy matrix (Fig. 1A,B).