Kinetics of adherence of mucoid and non-mucoid *Pseudomonas aeruginosa* to plastic catheters

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Summary. The adherence of six non-mucoid and three mucoid strains of *Pseudomonas aeruginosa* to polyvinyl chloride (PVC), polyurethane (PU) and siliconised latex (SL) was evaluated by a radiometric method and scanning electronmicroscopy. Initially greater numbers of mucoid than non-mucoid strains adhered to all three materials. Hydrophobic non-mucoid strains adhered more efficiently than hydrophilic strains. Numbers of adherent non-mucoid *P. aeruginosa* cells increased with time, reaching a peak, which was different for each strain, at 1–4 h for PU, 4 h–2 days for SL and 2–3 days for PVC; thereafter a gradual decrease was observed, maximal and final values of adherence being higher with PVC and SL than with PU. Adherence of mucoid strains increased with time in 3–5 days, until a steady state was reached. We conclude that although non-mucoid strains of *P. aeruginosa* initially colonise plastic surfaces better than mucoid strains, mucoid strains also persist on these surfaces.

Introduction

Pseudomonas aeruginosa is a common cause of infection in immunocompromised patients and those with cystic fibrosis.^{1,2} It is an important cause of hospital acquired infection such as urinary tract infection and pneumonia.³

P. aeruginosa can adhere to solid surfaces⁴ and colonisation of inanimate surfaces by micro-organisms depends on properties of the micro-organism and the environment.⁵ Initial adherence is considered to depend mainly on surface properties of bacteria, such as surface hydrophobicity and net surface charge. Synthesis of exopolysaccharide materials (slime) appears to be the most important factor for irreversible adherence of bacteria.⁴

Previous studies on bacterial adherence to catheters and other medical devices have been done with *Staphylococcus* spp. (mainly *S. epidermidis*),^{6,7} enterobacteria^{8,9} and *P. aeruginosa*.^{10,11,12} Most of these studies used electronmicroscopy, but no kinetic models have been established.

In this paper we describe a kinetic model of adherence for mucoid and non-mucoid strains of *P. aeruginosa* to three different materials—polyvinyl chloride, polyurethane and siliconised latex.

Materials and methods

Strains

Six non-mucoid (strains 1–6) and three mucoid

strains (7–9) of *P. aeruginosa* isolated from clinical sources at the Hospital Universitario de Sevilla, Spain, were used. Strains 1 and 5 were cultured from wounds, strain 2 from blood, strain 3 from pharyngeal exudate, strain 4 from bronchial aspirate, strain 6 from urine from a catheterised patient and strains 7–9 from sputum of patients with cystic fibrosis.

A non-mucoid variant of strain 9 was obtained by culturing the original strain in Tryptic Soy Broth (TSB) (Difco) at 37°C for 18 h (i.e., to the stationary phase), and subculturing on Tryptic Soy Agar (Difco) at 37°C for 48 h to isolate non-mucoid variants.

Strains were identified according to standard methods and by the API 20NE System (API System SA, France).

Heavy suspensions of each strain in TSB with glycerol 10% were stored in small volumes at –70°C.

Biomaterials

Catheters made of three different materials were assessed: polyvinyl chloride (PVC) (neonatal umbilical catheter, Argyle® 3·5, Sherwood Medical Industries Ltd, Belgium); siliconised latex (SL) (pediatric foley catheter, INMED®, Malaysia); and polyurethane (PU) (peripheral venous catheter, Cavafix Certo®; B. Braun Melsungen AG, FRG, kindly supplied by Palex, Spain). The catheters were cut into 1-cm lengths under sterile conditions.

Surface hydrophobicity

The method of Rosenberg *et al.*¹³ was used to determine surface hydrophobicity: 3 ml of phosphate-buffered saline (PBS), pH 7·2–7·4, containing 10⁹ cfu/

ml (measured by spectrophotometry—initial optical density, OD_i) was vortex-mixed for 1 min with 0.35 ml of *p*-xylene (Merck, FRG), left for 30 min to allow phase separation and the OD of the aqueous phase was again measured (final optical density, OD_f). Surface hydrophobicity was expressed as a percentage according to the formula:

$$\text{Surface hydrophobicity} = [1 - (OD_f/OD_i)] \times 100.$$

Adherence to catheters

Bacteria were grown in 5 ml of TSB containing (2— ^3H)-adenine (24 Ci/mmol) (Amersham) 10 μl for 18 h at 37°C. After centrifugation (5000g, 15 min, 4°C) bacteria were washed three times with 10 ml of cold PBS. A PBS suspension of each strain containing radiolabelled bacteria (10^8 cfu/ml) was prepared; 100 μl -samples of each suspension and 2.5 ml of scintillation cocktail were vortex-mixed for 1 min in polypropylene vials (Biovials®, Beckman, USA) and radioactivity was counted in a liquid scintillation system (Wallac 1210 Ultrobeta, LKB, Sweden). Three vials were counted for each suspension, and labelling efficiency was calculated by dividing the initial counts by the mean ($\times 10$) of the cpm values obtained.

Two pieces of catheter were used for each measurement at 5, 15 and 30 min, 1, 4 and 8 h, and 1–7 days. Catheter pieces were initially wetted by immersing for 1 h in 10 ml of PBS at 37°C and then transferred to 10 ml of labelled bacterial suspension in 25 ml screw-capped sterile glass bottles at 37°C. At each specified time two pieces of catheter were carefully removed and washed five times with cold PBS to remove non-adherent bacteria. The final washing was checked to ensure that it was free from radioactivity. Each piece was then placed in a polypropylene vial, vortex-mixed for 1 min with 2.5 ml of scintillation cocktail and counted as before. Values, in cpm, and numbers of adherent bacteria were calculated for each segment of catheter. The total surface area of each segment was calculated and final results were expressed as adherent cfu/cm² of material.

Table I. Surface hydrophobicity of *P. aeruginosa* strains.

Strain no.	Surface hydrophobicity—mean (SD) of three determinations
1	17.9 (2.4)
2	42.1 (0.4)
3	15.0 (0.6)
4	15.0 (3.5)
5	18.8 (2.0)
6	44.6 (6.4)
7*	23.0 (12.0)
8*	20.5 (1.8)
9*	22.4 (5.1)

*Mucoid strains.

Scanning electron microscopy (SEM)

Adherence of strain 2 to all three materials was studied at 5, 15 and 30 min and 1, 4, 8 and 24 h by SEM. After removing the pieces of catheter from the bottles they were fixed in paraformaldehyde 1% glutaraldehyde 10% for 3–4 h. After washing with PBS for 5 min, samples were treated for 20 min with osmium tetroxide 1% and dehydrated in increasing concentrations of ethanol (70%, 90%, 96% for 5 min each and 100% for 3 h). Pieces of catheter were dried by the CO₂ critical point technique, fixed on aluminium stubs, covered with a 300 Å gold film and observed by scanning electron microscopy (ISI-SX-25, Japan).

Statistical analysis

All the experiments were done in duplicate (except for SEM) and the mean value was calculated; where appropriate comparisons were made by Student's *t* test; values of $p < 0.05$ were regarded as significant.

Results

Surface hydrophobicity

Values obtained with the test strains ranged from 15.0 SD 0.6% (strain 3) to 44.6 SD 6.4% (strain 6). Two of the strains (2 and 6) were relatively hydrophobic (surface hydrophobicity $> 40\%$), the rest were relatively hydrophilic (surface hydrophobicity $< 20\%$). Tests with all three mucoid strains gave intermediate values: 20.5 SD 1.8% to 22.4 SD 5.1% (table).

Adherence of non-mucoid and mucoid strains to catheter materials

The kinetics of adherence are shown in fig. 1.

Adherence to PVC. The number of adherent bacteria of mucoid strains increased with time, reaching maximal adherence (MaxAdh), at 2 days (strain 7: 0.36×10^6 cfu/ml), 6 days (strain 8: 1.8×10^6 cfu/ml) and 7 days (strain 9: 1.2×10^6 cfu/ml).

Generally, initial adherence (5 min) to PVC was higher (10–30-fold) for non-mucoid (0.49 SD 0.14×10^6 cfu/cm²) than for mucoid strains (0.031 SD 0.027×10^6 cfu/cm²). There were significant differences in adherence between more hydrophobic (2 and 6) and more hydrophilic non-mucoid strains (1, 3, 4 and 5) after 60 min of interaction: 3.00 SD 0.80×10^6 cfu/cm² and 1.25 SD 0.41×10^6 cfu/cm² ($p < 0.01$). After 4 h, adherence was independent of surface hydrophobicity.

The number of adherent non-mucoid bacteria increased over 3 days, reaching MaxAdh between 2.4×10^6 (strain 4) and 19×10^6 cfu/cm² (strain 3). Strain 6 was the only non-mucoid strain that reached MaxAdh in 60 min.

Thereafter the number of adherent bacteria de-

Fig. 1. Kinetics of adherence of *P. aeruginosa* to PVC (■), PU (▨) and SL (□). (A), non-mucoid strains (6); (B), mucoid strains (3). Results are mean values of three experiments, bar=SD.

creased slowly; at 7 days the numbers of adherent bacteria ranged from 0.9×10^6 (strain 6) to 8.7×10^6 cfu/cm² (strain 2). MaxAdh values were significantly higher in non-mucoid ($8.8 \text{ SD } 6.7 \times 10^6$ cfu/cm²) than in mucoid ($1.1 \text{ SD } 0.7 \times 10^6$ cfu/cm²) strains ($p < 0.05$).

Adherence to PU. Mucoid strains were initially less adherent ($0.016 \text{ SD } 0.010 \times 10^6$ cfu/cm²) than non-mucoid ones ($0.49 \text{ SD } 0.35 \times 10^6$ cfu/cm²) ($p < 0.01$). MaxAdh was also lower with mucoid ($1.1 \text{ SD } 0.5 \times 10^6$ cfu/cm²) than with non-mucoid strains ($3.3 \text{ SD } 1.6 \times 10^6$ cfu/cm²) ($p < 0.01$), but final adherence was higher in mucoid strains ($1.0 \text{ SD } 0.6 \times 10^6$ cfu/cm²) than in non-mucoid strains ($0.35 \text{ SD } 0.1 \times 10^6$ cfu/cm²).

Differences between hydrophilic and hydrophobic non-mucoid strains were significant only in the first

5 min: $0.28 \text{ SD } 0.16 \times 10^6$ and $0.90 \text{ SD } 0.14 \times 10^6$ cfu/cm² respectively ($p < 0.01$). MaxAdh was reached in only 4 h with non-mucoid strains and was 2.7-fold lower than that observed with PVC. Adherence decreased slowly, reaching a minimum at 7 days.

Adherence to SL. Mucoid strains adhered less efficiently ($0.017 \text{ SD } 0.012 \times 10^6$ cfu/cm²) than non-mucoid strains ($0.41 \text{ SD } 0.28 \times 10^6$ cfu/cm²) ($p < 0.01$) and final numbers of adherent bacteria ($1.2 \text{ SD } 0.8 \times 10^6$ and $2.9 \text{ SD } 0.6 \times 10^6$ cfu/cm²) were also significantly lower with mucoid than non-mucoid strains.

There were significant differences at 5 min between hydrophilic and hydrophobic non-mucoid strains ($0.29 \text{ SD } 0.14 \times 10^6$ and $0.72 \text{ SD } 0.18 \times 10^6$ cfu/cm²) ($p < 0.05$) and MaxAdh was reached in 1–4 h with hydrophobic strains and 1–2 days with hydrophilic strains.

Fig. 2. Adherence of a non-mucoid variant of *P. aeruginosa* (A) and its parental mucoid strain (B) to PVC (□—□) and PV (●—●).

Fig. 3. Adherence of a non-mucoid strain of *P. aeruginosa* to PVC: A, 5 min; B, 60 min; C, 24 h ($\times 10000$; bar = 1 μm) D, Adherence to PU at 24 h ($\times 2500$; bar = 4 μm).

Adherence to PVC and PU of a non-mucoid variant from a mucoid strain

A non-mucoid variant of strain 9 had a similar kinetic model with PVC and PU to that observed in

initially non-mucoid strains 1–6. At 5 min, adherence to PVC (0.17×10^6 cfu/cm²) and PU (0.19×10^6 cfu/cm²) was 17- and 19-fold higher than with the original mucoid strain (0.01 and 0.098×10^6 cfu/cm²).

MaxAdh for this non-mucoid variant was reached

at 2 days (PVC) and 4 h (PU), in contrast to 7 days with the mucoid parent (fig. 2).

SEM

Fig. 3 shows the adherence of strain 2 to PVC and PU with time. At 5 min (Fig. 3A) a few micro-organisms appear on PVC, some of them occupying irregularities on the surface; after 1 h, the number of adherent bacteria increased, and an amorphous material was evident around the micro-organisms (fig. 3B); at 24 h slime could be seen covering a multilayer bacterial biofilm (fig. 3C). Similar pictures were obtained with SL catheters (not shown). Adherence to PU was similar at 5 and 60 min but at 24 h the bacteria formed a single layer of cells with no sign of slime production (fig. 3D).

Discussion

Colonisation of medical devices by *P. aeruginosa* is likely to depend on the ability to adhere to solid surfaces^{4,10,14} which then allows micro-organisms to form biofilms in which they are protected from harmful environmental factors.⁴

Most studies of microbial factors involved in adherence to inert surfaces have focused on physical forces such as surface hydrophobicity. Our strains were neither strictly hydrophobic nor strictly hydrophilic. Our results agree with those of Speert *et al.*¹⁵ who obtained values ranging from 15% to 65% with a polyethyleneglycol-dextran system. Previous reports have shown that hydrophobic micro-organisms adhere more efficiently to hydrophobic substrata than hydrophilic ones.^{4,7,16-18} Our results confirmed that hydrophobicity of non-mucoid strains of *P. aeruginosa* was important during the first 5 min of interaction with catheters. Surface hydrophobicity did not appear to be important thereafter. Mucoid strains had intermediate surface hydrophobicity values, but initial adher-

ence to all three types of catheter was lower than for non-mucoid strains with similar surface hydrophobicity.

Different patterns of adherence to plastics were observed with non-mucoid and mucoid strains. With non-mucoid strains, adherence increased throughout the time reaching a peak (MaxAdh) quantitatively different for different strains. After MaxAdh was reached, the adherent population decreased slowly during the study period (7 days). Adherence of mucoid strains slowly increased until a steady state was reached (Fig. 1).

The difference in behaviour of mucoid and non-mucoid strains was confirmed by comparing the adherence of a mucoid strain and its non-mucoid variant to PVC and PU.

The production of slime has been shown to be important for colonisation of both medical devices^{2,19} and cell surfaces.^{20,21} Slime can also protect *P. aeruginosa* from antimicrobial drugs^{22,23} and host defence mechanisms.^{24,25} The mechanisms involved in mucoid-non-mucoid reversion are only partially understood but the recent finding of polyuronic-depolymerase activity in *P. aeruginosa*²⁶ and the recognition of slime production by "non-mucoid" strains²⁷ are significant. Our electronmicrographs confirmed that synthesis of a slime-like material was involved in adherence of non-mucoid strains. This slime-like material is known to consist of three chromatographic fractions, one of them being uronic acid, as in the slime produced by mucoid strains.^{27,28}

Adherence values were also influenced by the nature of the material especially with non-mucoid strains. These differences may justify in-vitro studies of micro-organism-material interactions when evaluating the application of new materials for clinical use.

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