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Adherence of *Pseudomonas aeruginosa* to inanimate polymers including biomaterials

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Cells of *Pseudomonas aeruginosa* were adhered to polymethyl methacrylate, polyvinyl acetate, polyvinyl chloride, polyhydroxyethyl methacrylate, mixed-acrylic, silicone, and natural latex materials. Planktonic bacteria and bacteria that adhered to the test materials were compared for their uptake of either L-[3,4,5-^{3}H] leucine or [*methyl*-^{3}H] thymidine during growth in a minimal medium. Leucine incorporation was reduced and thymidine uptake was negligible in adherent bacteria for up to 8 h following primary attachment by which time cells in the planktonic state showed active uptake of both substrates. These reduced uptake periods correlated with lag phases of growth of adherent cells as determined with a sonication-release plate count procedure and analyses of adenosine triphosphate (ATP). The extent of the lag phase of the adherent populations was dependent on initial densities of adhered cells and the nature of the substratum.

Keywords: adherence; *Pseudomonas aeruginosa*; polymers

Introduction

Bacteria *in vivo* colonize a variety of medical devices including contact lenses, intraocular lenses, intrauterine devices, and various types of catheters and stents (ie, urinary, peripheral, central venous). These colonizations often result in device-related infections that are an increasing cause of hospital and community morbidity and mortality [14,15,23]. Bacteria on the devices are frequently found in secreted slime matrices that are often difficult to penetrate with antimicrobial agents [1,6,16]. Organisms residing in these secreted slime layers are typically more resistant to antimicrobial agents than bacteria existing in the planktonic state [2,17,18,20,26]. Whether the enhanced resistance is primarily due to protection conferred by the secreted glycocalyx or to lower growth rates which have been observed for biofilm bacteria, is not clear [5,8,30]. The possibility also exists that the formation of a resistant biofilm matrix may be consequential to an initial change in the bacterial cell metabolism following the adhesion process and prior to formation of a protective slime layer, ie substratum-induced changes in newly adhered cells caused by activation of specific genes [7,9]. Therefore, the attachment of microorganisms to surfaces may influence expression of genes involved in resistance to antimicrobial agents and to other phenomena (eg surface growth and accumulation kinetics) that affect subsequent development of a mature biofilm [3,11,12].

In our laboratories, *Pseudomonas aeruginosa* No. 3 has been employed for evaluation of surfaces (contact lenses, intraocular lenses, urinary catheters and stents) for their relative resistance to primary adherence by bacteria [13,21,22,27]. Additionally, the strain has been used in determining the relative efficacy of antimicrobials including bound inhibitors [19,24]. In this report, we examine factors affecting *in vitro* primary adherence and subsequent accumulation of *P. aeruginosa* No. 3 on inanimate surfaces.

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Materials and methods

Cultivation and maintenance of bacteria

Pseudomonas aeruginosa No. 3, a slime-forming isolate recovered from a corneal ulcer [20] and shown previously to adhere readily to contact lenses [21] and urinary catheters [13], is maintained in the lyophilized stock culture collection at Georgia State University. Working cultures were maintained on tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA) Slants were stored at 4°C and subcultured monthly. Prior to experimentation, the identity of each culture recovered from lyophilization was confirmed for specific colony type and for compatibility with the species with the API 20E and Vitek systems (bioMerieux Vitek, Inc, Hazelwood, MO, USA).

Materials

Silicone (SI) was obtained from sterile Foley catheters that were sectioned aseptically above the balloon and below the distal end into 5-mm lengths. The natural latex (LA) samples were 9-mm diameter grommets used for catheter sampling ports. The samples of mixed-acrylic (ethylmethyl, MA), polymethyl methacrylate (PMMA), and hydrated polyhydroxyethyl methacrylate (polyHEMA, 58% water, w/w) materials were entire intraocular lenses and contact lenses. The polyvinyl acetate (PVA) and polyvinyl chloride (PVC) surfaces (10 mm^2 coupons) were industrial grade materials. All surfaces except the polyHEMA hydrogel were hydrophobic. None of the test materials possessed an antimicrobial surface coating. Catheter and lens materials were sterile and were processed aseptically. Industrial grade materials were rinsed with 70% ethanol.

All materials were handled with powder-free sterile gloves. Hydrogel contact lenses were handled with Teflon-coated forceps.

Primary adherence

The relative adherences of *P. aeruginosa* to the test materials were determined with modifications of the procedures of Sawant *et al* [27] and Gabriel *et al* [13]. Cells were grown overnight (18 h) at 37°C in tryptic soy broth (TSB, Difco) on a rotary shaker (150 rpm). The cells were harvested in a centrifuge at $5000 \times g$ for 10 min and washed twice with 0.9% saline and adjusted to a density of about 10^8 cells ml^{-1} in phosphate-buffered saline (PBS; 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter of deionized water; pH 7.4). In general, test items were introduced into 3 ml of the cell suspension in sterile 20-ml glass scintillation vials which were incubated for 2 h at 37°C. The materials were removed aseptically and rinsed five times in each of three successive volumes (200 ml) of PBS. Samples of each material were transferred separately to vials containing 3 ml of minimal broth (7.0 g K_2HPO_4, 2.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 0.5 g sodium citrate, and 1.0 g d-glucose in 1 L of deionized water). Materials were incubated at 37°C for the indicated sampling intervals prior to leucine and thymidine uptake assays, ATP extraction, or sonication-release.

Leucine uptake

Prelabeled cells: The cell suspensions in PBS were incubated for 1 h at 37°C in shaken cultures followed by the addition of 0.05% volume of l-[3,4,5-^{3}H] leucine (NEN Research Products, DuPont Company, Wilmington, DE, USA) and then were incubated for an additional 20 min. These cells were washed four times in PBS and adjusted to 10^8 cells ml^{-1} in PBS or minimal broth. Test items were incubated in 3 ml of radiolabeled cell suspension for 2 h, rinsed (immersed five times in each of these successive changes of saline, 200 ml) and then placed into 10 ml of Opti-Flour cocktail (Packard Instrument Co, Downers Grove, IL, USA) in scintillation vials. Vials were mixed with a vortex mixer and radioactivity was measured in a liquid scintillation counter (LS-7500 Beckman Inst, Fullerton, CA, USA).

Postlabeled cells: The uptake of leucine by adhered cells from minimal medium was determined at 4, 8, 12, 16 and 24 h. Test materials with adhered bacteria were transferred after the rinsing step to vials containing fresh minimal medium supplemented with 2 μCi ml^{-1} of l-[3,4,5-^{3}H] leucine. These samples were incubated for 30 min at 37°C on a rotary shaker. Following the radiolabeling step, the materials were rinsed as per the prelabeled cells and each sample was placed into 10 ml of Opti-Fluor cocktail and radioactivity was determined as described above. Planktonic cells from the minimal broth were run in parallel for their leucine uptake and dilutions of the inocula in each experiment were radiolabeled with leucine and enumerated on tryptic soy agar (TSA, Difco). Data were reported as colony forming units mm^{-2} for the 2-h incubation period and as dpm mm^{-2} and dpm ml^{-1} for sessile and planktonic bacteria, respectively, for the other time periods.

Thymidine uptake

Methyl thymidine uptake by cells of *P. aeruginosa* was determined with a modification of the methods of Davies and McFeters [10]. Test materials were placed into minimal media supplemented with 1 μCi ml^{-1} of [methyl-^{3}H] thymidine (NEN Research Products) with a specific activity of 6.70 Ci mM^{-1}. The materials were incubated at 37°C on a rotary shaker for the indicated time intervals up to 12 h. Every 2 h, representative samples were removed from the broth, rinsed, and transferred to scintillation cocktail and evaluated for radioactivity. In both the leucine and thymidine uptake assays, uninoculated test materials were incubated with the radiolabeled substrates and results were adjusted for non-metabolic adsorption of the leucine or thymidine.

Bioluminescence (ATP extraction)

Test materials with adhered bacteria were rinsed and transferred to wells of a 24-well polystyrene culture plate (Corning Glass Works, Corning, NY, USA) containing 0.5 ml of PBS. A nonionic surfactant (0.5 ml Extractant-XM, Biotrace, Inc, Plainsboro, NJ, USA) was added (with mixing) to each well. After 5 min, the plates were agitated gently and 0.2 ml from each well was dispensed into luminometer cuvettes. A 0.1-ml volume of reconstituted luciferase-luciferin reagent (Enzyme-MLX, Biotrace) was added to each cuvette and the reaction mixture was analyzed in a luminometer (Uni-Lite X-cel, Biotrace). The relative light units (RLUs) or light output were converted to cell numbers with a calibration curve derived from actual cell numbers and adjusted for any nonspecific luminescence generated by uninoculated substrata.

Sonication-release

Test materials with associated bacteria were removed from the vials after 0, 6, 16 and 24 h and rinsed in three successive changes of PBS. Then each sample was placed in a separate sterile vial with 1 ml of PBS and sonicated for 1 min at 75 kHz in an ultrasonic bath (Branson Ultrasonics Corp, Danbury, CT, USA) filled to a 1-cm depth with deionized water. The vials were vortexed and adhered bacteria released from test samples were enumerated on TSA spread plates incubated for 24 h at 37°C. Data were expressed as CFU mm^{-2} for all test surfaces.

Statistical analyses

All tests for sonication-release, leucine uptake, thymidine uptake, and analysis of ATP were conducted in sets of five for each type of substratum at a given sampling interval. Most experiments were performed at least in duplicate. Data from each test group were plotted linearly and compared statistically (Sigma Plot for Windows Version 4.00, Jandel Scientific, Sausalito, CA, USA) ($P < 0.05$).

Results

The growth rate of planktonic cells of *P. aeruginosa* in minimal broth (0.46^{-h}) exceeded the rates of accumulation

of adhered irreversibly bound cells on the MA (0.16^{-h}), PMMA (0.26^{-h}), and SI (0.27^{-h}) materials suspended in the medium (Figure 1). Adhered cell numbers at 2 h differed for the various surfaces and showed only slight increases on all surfaces for 6–8 h, by which time cells in the planktonic state were in the logarithmic phase of growth. At the 2–4 h intervals, the numbers of cells adhering from minimal medium and PBS to a given substratum were similar and exceeded those adhering from TSB (data not shown). There was a trend for fewer cells to be released by sonication from MA than from the other surfaces. Numbers of adhered cells were essentially constant after 15 min to 4–6 h even when the rinsing procedure was repeated (six volumes). Scanning electron microscopic observations showed a few cells (estimated at 10–100 cells mm^{-2}) remaining on all substrata after the rinsing or rinsing-sonication procedure (data not shown).

Compared with cells in the planktonic state, the cells adhered to surfaces (particularly LA and MA) showed reduced to negligible capacity for l-leucine uptake during the first 8 h after contact with the surface (Figure 2). These data correlated in general with the increase in cell numbers with time that were released from the surfaces by sonication. In separate agar diffusion tests of all materials, zones of inhibition were present only with some of the LA samples. It is assumed that soluble inhibitors accounted for the depression of leucine uptake by cells on the latex. Thymidine uptake by planktonic cells was immediate, appreciable, and tracked the growth curve for 2–4 h, whereas thymidine uptake by adhered cells was depressed during the first 6–8 h after attachment (Figure 3). The uptake of thymidine by cells adhered to PVA and PVC occurred earlier than with cells adhered to LA and SI.

Accumulation of irreversibly-bound cells on three different materials was measured as total ATP extracted from the adhered cells and expressed in relative light units (RLUs) (Figure 4). The ATP content of cells adhered to SI, from an inoculum of 3×10^6 cells was almost 100-fold higher than from cells on MA and PMMA. The ATP content from cells on MA and PMMA initially decreased with time but then increased at a higher rate than for cells on silicone. The relative adherence of cells to MA and PMMA for 4 h and longer, as indicated by ATP content, appeared inverse to that indicated by the uptake of leucine and thymidine. The MA samples, however, for ATP analysis differed from those used in the radiolabel studies in that the latter had been disinfected with H_2O_2. We have not investigated this aspect further. When different densities of cells were adhered to silicone, the ATP content with time increased at a lower rate with the higher inoculum (Figure 5). This

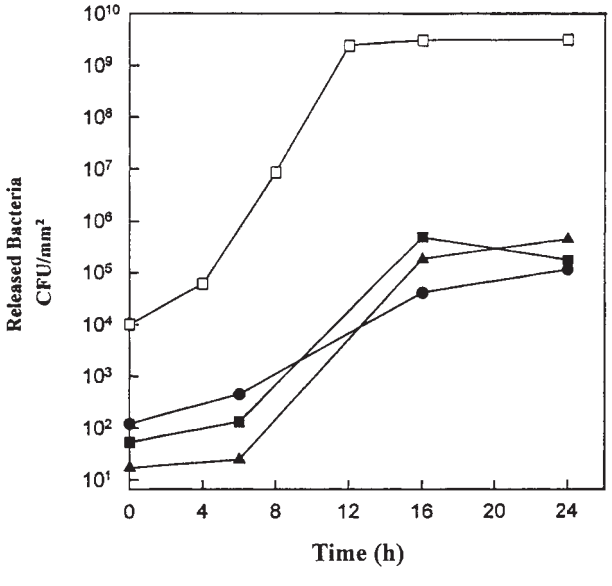

Figure 1 Densities of cells of *P. aeruginosa* adhered to hydrophobic polymers: mixed acrylic (MA, ●); polymethyl methacrylate (PMMA, ■) and silicone (SI, ▲) from minimal medium *vs* cell densities in the minimal medium (MM, □). Differences in all non-overlapping data points were significant $n = 5$ ($P \leqslant 0.05$).

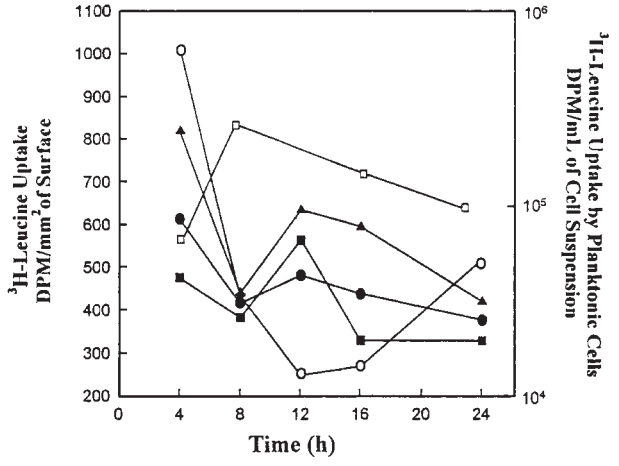

Figure 2 Uptake of tritiated-leucine by cells of *P. aeruginosa* adhered on MA (●), PMMA (■), SI (▲), and LA (○) *vs* planktonic cells (□). Differences in all non-overlapping data points were significant $n = 5$ ($P \leqslant 0.05$).

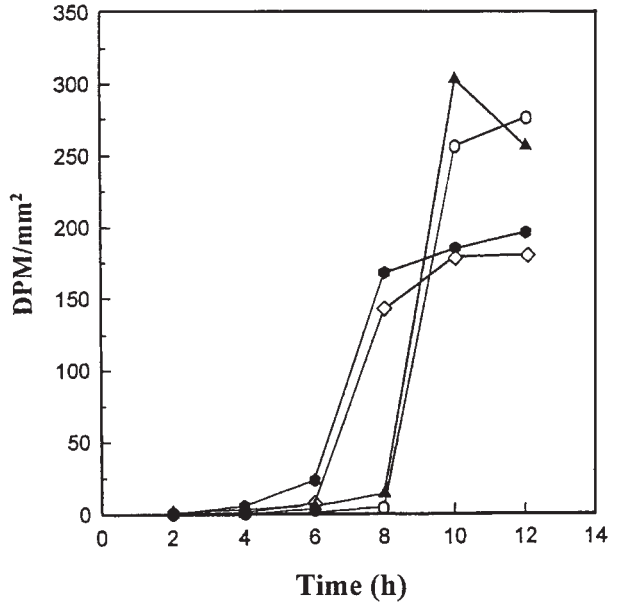

Figure 3 Uptake of ^{3}H-methyl thymidine by *P. aeruginosa* adhered to SI (▲), LA (○), PVA (◆), and PVC (◇). Differences in all non-overlapping data points were significant $n = 5$ ($P \leqslant 0.05$).

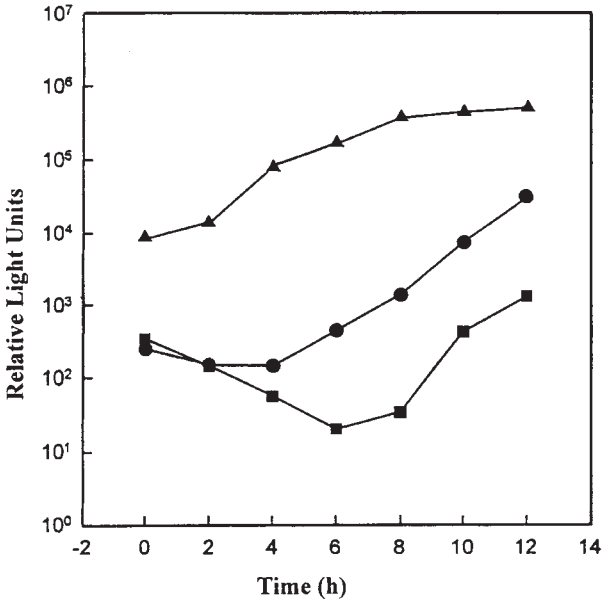

Figure 4 ATP content expressed as relative light units (RLU) of *P. aeruginosa* on SI (▲), MA (●), and PMMA (■). Differences in all non-overlapping data points were significant $n = 5$ ($P \leq 0.05$).

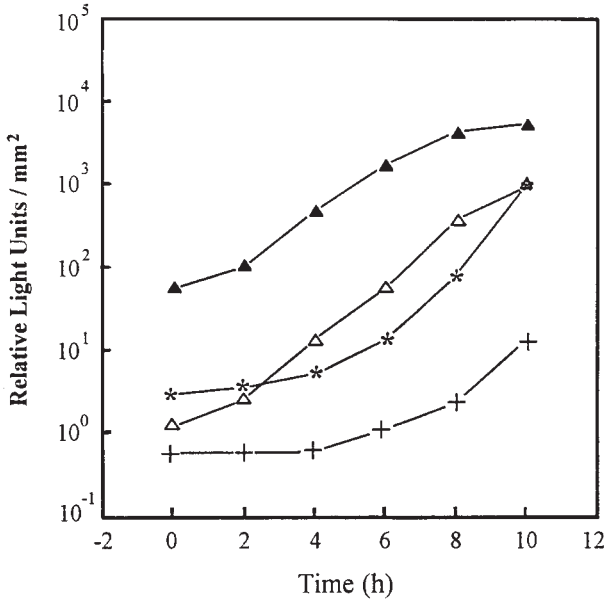

Figure 5 Increase with time in ATP content of *P. aeruginosa* adhered to Si (△, 10^6 cells m^{-1}; ▲, 10^7 cells ml^{-1}) and polyHEMA (+, 10^6 cells ml^{-1}; *,10^7 cells ml^{-1}) from varying initial cell densities in MM. Differences in all non-overlapping data points were significant $n = 5$ ($P \leq 0.05$).

phenomenon was not evident with cells adhered to polyHEMA but differences in degrees of adherence to substrata of varied hydrophobicity were again evident and significant. The bubble contact angle of pure water on polyHEMA was zero, whereas the contact angle on the silicone was greater than 100°.

Discussion

The degree of primary adherence of *P. aeruginosa* No. 3 from PBS or MM to various biopolymers varied with the type of polymer and with the density of cells in the planktonic state. The rate of accumulation of irreversibly bound cells on a given surface tended to be lower with the higher inocula. In our studies, surface areas of about 160–460 mm^2 of selected polymers with relatively smooth surfaces (determined by scanning electron microscopy, data not shown) were exposed from 3×10^6 to 3×10^8 total cells in PBS or MM. Maximal primary adherence after 2 h was about 1×10^5 cells mm^{-2} found for SI, PVA and PVC. At these concentrations the cells were not coalesced into a biofilm although sparse clumps of cells were present. Usually there was at least one log fewer cells adhering to polyHEMA compared to the hydrophobic surfaces.

During the first 6–8 h, a reduced or negligible uptake of thymidine and leucine by adhered cells of *P. aeruginosa* No. 3 suggested that primary adherence to the solid surfaces induced an extended lag phase compared to the lag phase for cells in the plankton. Decreased metabolic activity following primary adherence was indicated further by an essentially constant ATP level prior to an increase in the number of irreversibly bound cells. Bright and Fletcher [4] gave evidence that supported the existence of a direct substratum influence on the assimilation of amino acids in a marine *Pseudomonas* sp. Cells adhered to polytetrafluoroethylene demonstrated higher V_{max} values than free-living cells when leucine was the substrate. The densities of adherent cells of Gram-negative bacteria (*Pseudomonas*, *Vibrio*, and *Photobacterium* spp) also have been related to a quorum-sensing effect [28]. This probable quorum effect was supported further by Davies *et al* [11] who reported that a strain of *P. aeruginosa* that lacked the ability to produce an acylated homoserine lactone failed to produce a mature biofilm with mounded colonies. *P. aeruginosa* No. 3 in minimal medium produced a mature biofilm within 48–120 h dependent upon the substratum. Our data for the first few h of cell contact with inanimate surfaces indicate a lag period in accumulation of firmly adhered cells that varied, in part, upon the initial adherent cell densities and the nature of the substratum. A differential effect on metabolic activity by substrata of essentially identical hydrophobicities (Figure 3, PVA and SI with bubble contact angles $>100^0$) has not been reported for non-antimicrobial surfaces. Whether the end of the lag periods was associated with synthesis of homoserine lactone is not known.

Substratum characteristics such as surface charge and hydrophobicity as well as the physiological properties of the strain are known to affect the extent of irreversible primary adherence of strains of *P. aeruginosa* to biomaterials [20,23]. Adsorption of macromolecules onto substrata may also influence the amount of bacterial adherence [22,25]. Attachment to solid surfaces has been reported to immediately upregulate alginate synthesis in a strain of *P. aeruginosa* [9], therefore strengthening cell-substratum binding. Recently, Tang and Cooney [29] reported that biofilms of *P. aeruginosa* PA 0-1 on non-coated stainless steel contained a greater proportion of viable cells than biofilms on aluminum or fiberglass. Moreover, antimicrobial paints were more effective at inhibiting early stage biofilm development (<6 days) on aluminum than on the other two substrata coated with the same two paints. The data indicated a

substratum effect on adherence although biofilm formation eventually appeared equivalent on all painted and unpainted surfaces. The substratum also showed an effect on the adherence of *P. aeruginosa* No. 3 although eventual attainment of equivalent adhered numbers on the different substrata (although implied) was not demonstrated.

Biomaterials that demonstrate low primary adherence may be significant for *in vivo* usage. Reduced primary adherence and prolonged lag phases could permit more effective immune responses. This suggests that the administration of antibiotics in device implantation procedures immediately after surgery may be of lesser value than their administration preoperatively or postoperatively (ie after several hours). These speculations are worthy of consideration in the implantation of certain high risk devices.

Considerable interspecies and intraspecies strain variation has been found among various bacteria and for strains of *P. aeruginosa* in adherence studies [6,9,28]. Our current work involves a single strain of *P. aeruginosa* that was selected from 15 strains that showed lesser and more indiscriminate adherence. Whether our observations with a strain of *P. aeruginosa* apply to the primary adherence to biomaterials by strains of other species has yet to be established.

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