The effects of adherence to silicone surfaces on antibiotic susceptibility in Staphylococcus aureus

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Sensitivity of Staphylococcus aureus to the antibiotics tetracycline, benzylpenicillin and vancomycin was found to decrease by 2–10-fold when cells were grown adherent to silicone catheter surfaces. Sensitivity to rifampicin and fusidic acid was not significantly altered in adherent cells. Susceptibility further decreased with increased adherence time prior to antibiotic challenge. The resistance observed was not genotypic, or due to the presence of a specialized subpopulation of bacteria, as it disappeared when the bacteria were removed from the catheter, subcultured and retested. Also, adherent bacteria were found to grow more slowly than bacteria growing planktonically. It is concluded that the decrease in antibiotic susceptibility of adherent bacteria is a function of the physiological status of the individual cells rather than a function of biofilm formation or slime production. The decrease in growth rate of the adherent bacteria is a result of the adherence process rather than a result of nutrient depletion. The decrease in growth rate is implicated, but is not the sole factor, in the decreased antibiotic susceptibility of adherent bacteria.

Keywords: Staphylococcus aureus, antibiotic susceptibility, adherence

INTRODUCTION

Staphylococcus aureus is a significant pathogen causing infections, usually in compromised individuals such as the immuno-suppressed, malnourished or those with implanted foreign bodies such as indwelling catheters or prosthetic devices. Diseases caused by S. aureus include osteomyelitis, endocarditis, meningitis, food poisoning and toxic shock syndrome (Sheagren, 1984). S. aureus commonly colonizes artificial airways (Cross & Roup, 1981), ocular prostheses (Chalupa et al., 1987), orthopaedic devices such as replacement hip and knee joints (Pugsley & Sanders, 1987), and intravascular (Dickinson & Bisno, 1989) and peritoneal dialysis (Luzar et al., 1990) catheters. Catheter-associated infections are often difficult to treat owing to their decreased susceptibility to antibiotic therapy. However, when causative bacteria are tested in vitro they are often found to be susceptible to the same antibiotic to which they showed decreased susceptibility in vivo (Craddock et al., 1987). The mechanism of such decreased susceptibility in vivo is not understood but is thought to be a consequence of surface-attached growth. For this reason, much attention has recently been focused on investigation of the mode of growth in vivo, particularly surface-attached growth.

Bacteria stick tenaciously and often with great specificity to a wide range of surfaces and this property seems to be of great importance in the initiation of an infection. When growing on surfaces, bacteria produce an extracellular polysaccharide layer, the glycocalyx. The glycocalyx surrounds the individual bacteria, which form microcolonies that ultimately coalesce to form a confluent biofilm (Costerton et al., 1987). Surface-growing bacteria are profoundly different from planktonic bacteria, both physiologically and morphologically. When growing on surfaces, bacteria exhibit many changes such as alteration of the nature of their surface proteins (Cheung & Fischetti, 1988), production of β-haemoly-
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sins (Lorian, 1971) and formation of unusually thick cell walls and cross walls (Lorian et al., 1985). In addition, altered metabolic activity (Marshall, 1976; Hattori & Hattori, 1976; Gordon et al., 1983) and differences in growth rates (Brown et al., 1988) and in substrate assimilation (Bell & Allbright, 1982) have been found between bacteria growing in suspension and on surfaces.

It has been suggested that the decreased antibiotic susceptibility of attached bacteria may result from the glycocalyx providing a barrier that reduces penetration of antibiotics. However, this view is not supported by the finding that diffusion to the bacterial surface is only minimally retarded in biofilms (Steckelberg et al., 1989; Dunne et al., 1993).

Slow growth is characteristic of many infections (Zak & Sande, 1981) and of bacteria growing within a biofilm on prosthetic devices (Brown et al., 1988). Coupled with the observations that non-growing or slow-growing bacteria show decreased susceptibility to a variety of antibiotics (Tuomanen, 1986), this suggests that growth rate may be a contributory factor to the decreased susceptibility of the attached cells. Restricted availability of nutrients is also a characteristic of bacterial growth during infections (Zak & Sande, 1981), and the nutrient-depleted growth conditions found in vivo can result in radically different cell wall compositions. This could also affect antibiotic susceptibility (Hugo & Davidson, 1973).

The present study compares the growth and antibiotic susceptibility of S. aureus growing planktonically and attached to silicone discs cut from Foley catheters. Results show that young adherent cultures that comprise single well-spaced cells can show significant decreases in antibiotic susceptibility. This indicates that biofilm formation is not essential for decreases in antibiotic susceptibility in adherent bacteria.

**METHODS**

**Bacterial strain.** The strain used was *Staphylococcus aureus* NCTC 8325-4, which is completely susceptible to all antibiotics as defined by the National Committee for Clinical Laboratory Standards (1984).

**Media and chemicals.** Growth media used were Mueller–Hinton broth (MHB), Tryptone Soya broth (Oxoid) and Tryptone Soya agar (Oxoid). Buffer used was 20 mM HEPES adjusted to pH 7.4 with HCl. Benzylpenicillin, rifampicin, tetracycline, vancomycin and fusidic acid were all supplied by Sigma. Catheters were supplied by Bard.

**Estimation of bacterial growth rate for planktonic cultures.** One colony of *S. aureus* was inoculated into 30 ml MHB and incubated for 18 h at 37°C. This was diluted 10-fold and 1 ml was added to 30 ml MHB. This was then incubated at 37 °C and samples of 0.1 ml were removed every hour for viable counts. These were performed by diluting in 20 mM HEPES (pH 7.4) and plating measured volumes to Mueller–Hinton agar (MHA) plates. Colonies were counted after 18 h incubation at 37 °C.

**Estimation of bacterial growth rate for adherent cultures.** Eight catheter discs (each of area 0.5 cm²), were inoculated with adherent bacteria as described below and placed in separate wells of a multiwell dish containing 2 ml MHB and incubated at 37 °C. At 15 min intervals, the discs were removed, rinsed in pre-warmed broth and transferred to wells containing fresh pre-warmed broth. Bacteria that had been shed from the catheter were estimated by performing viable counts on the broth from which the catheter had been removed. To estimate adherent bacteria, single discs were removed at 1 h intervals, sonicated, and viable counts were performed on the resulting suspensions. To determine the growth rate of the adherent bacteria, the numbers of bacteria attached to the disc surface were added to the numbers that had been shed into the supernatant, thus giving the total bacterial numbers. If it is assumed that most bacteria appearing in the supernatant do so as a result of growth and division of attached cells, changing the broth at 15 min intervals will preclude division of detached cells before they are counted.

**Determination of antibiotic susceptibilities.** Minimum inhibitory concentration (MIC) determinations were performed in duplicate on planktonic bacteria by the MIC microdilution method (National Committee for Laboratory Standards, 1984). The MIC corresponded to the antibiotic concentration in the first well to show no turbidity. After reading the MICs, the microtitre plates were placed on a microtitre shaker to resuspend the bacteria. A defined volume (10 µl) was transferred to an MHA plate and incubated at 37 °C for 24 h. The minimal bactericidal concentration (MBC) corresponded to the antibiotic concentration in the first well to show no growth on MHA. MIC/MBC determinations were also carried out as above but with 0.5 cm² of silicone disc placed in each well to ensure that the presence of catheter material did not affect the efficacy of the antibiotic.

**Evaluation of the effects of antibiotics on attached bacteria.** This method was adapted from La Tourette-Prosser et al. (1987). *S. aureus* was grown for 24 h on MHA at 37 °C, sonicated at 50 kHz for 5 min in a Dawe sonicleaner type 622A and suspended in 20 mM HEPES buffer (pH 7.4) to OD₅₅₀ 0.6 (Pye Unicam SP-840 spectrophotometer; path length 1 cm). Aseptically dissected catheter discs were placed in the suspension and incubated at 37 °C for 2 h to allow attachment of the bacteria. The catheter discs with adherent bacteria were used to produce two different types of culture: (1) to produce adherent cultures with well-spaced adherent bacteria (i.e. not forming a biofilm), the discs were incubated at 37 °C for a further 24 h in MHB; (2) to produce adherent cultures with discontinuous areas of biofilm, the discs were transferred to MHB and incubated at 37 °C for 7 d, changing the MHB every 48 h to prevent build-up of toxic metabolites.

Disc cultures of either type were then washed in 20 mM HEPES buffer (pH 7.4), transferred to MHB with or without antibiotic, incubated at 37 °C and removed at time intervals of 0, 2, 6, 24 and 48 h.

To estimate culture survival, discs were placed in 5 ml HEPES buffer, sonicated for 5 min, vortexed for 1 min to remove the bacteria and viable counts were performed on the buffer suspensions.

**Evaluation of the effects of antibiotics on planktonic cultures.** A single colony of *S. aureus* was inoculated into 30 ml MHB and incubated for 18 h at 37 °C. One millilitre of this was diluted 10-fold and added to 30 ml MHB containing antibiotic. This was then incubated for 48 h at 37 °C; 0.1 ml samples were removed at time intervals of 0, 2, 6, 24 and 48 h, and viable counts were performed.

**Scanning electron microscopy.** Catheter discs with adherent cultures were fixed at 4°C in 3% glutaraldehyde solution
**Table 1.** MICs and MBCs of *S. aureus* NCTC 8325-4 growing in suspension in MHB at 37 °C

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg ml⁻¹)</th>
<th>MBC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>1.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>0.062</td>
<td>0.125</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.015</td>
<td>0.031</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.125</td>
<td>0.250</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.015</td>
<td>0.250</td>
</tr>
</tbody>
</table>

(pH 7-4) for 1 h, then left for 24 h in phosphate buffer (pH 7-4). Discs were cut in half, postfixed in Millonig's phosphate-buffered osmium tetroxide at 4 °C for 1 h, then dehydrated through an ethanol series (50 %, 70 % and 100 %, v/v, ethanol; 15 min each) and critically point dried at 1200 bar pressure at 40 °C using liquid CO₂. Samples were mounted on aluminium stubs, gold sputter coated and viewed on a JEOL 5200 scanning electron microscope.

**RESULTS**

**Antibiotic susceptibility of planktonic *S. aureus***

Antibiotics were selected to cover a range of modes of action. MICs and MBCs were determined by the MIC microdilution method (National Committee for Laboratory Standards, 1984). Results shown in Table 1 indicate a high susceptibility to all antibiotics used.

**Structure of adhered cultures***

Cultures of *S. aureus* adherent to catheter discs were examined by scanning electron microscopy, firstly after incubation in MHB for 24 h, and secondly after growth in MHB for 7 d at 37 °C. After 24 h incubation, the attached culture comprised single, well-spaced adherent bacteria (Fig. 1a), whereas after 7 d incubation in MHB the attached culture, although still comprised largely of single cells, also included discontinuous areas of biofilm (Fig. 1b). These two cultures are referred to subsequently as adherent and biofilm, respectively. After sonication and vortexing to remove bacteria, the catheter discs were examined under the scanning electron microscope. This showed that almost all bacteria (92 %) were removed by this treatment.

**Growth of adherent bacteria in the absence of antibiotic***

Growth rate was estimated by adding numbers of detached and adherent bacteria as described in Methods. The resulting growth curves for the adherent bacteria are shown in Fig. 2. Neither culture appeared to grow exponentially but the adherent bacteria appeared to grow more slowly than planktonic cultures. The number of bacteria detaching slowly increased with time, suggesting that detachment was growth-related rather than the result of existing bacteria simply detaching from the surface.

**Effects of antibiotics on adherent, biofilm and planktonic *S. aureus***

Planktonic, 24 h adherent and 7 d biofilm cultures were all exposed to antibiotic at concentrations of 1 x, 2 x and 10 x MBC concentrations for 48 h. The percentage kills are summarized in Table 2. In most cases, results are given only for the 24 h time-point, but data for other time-points are included where significant.

**Vancomycin.** At all three antibiotic concentrations, the adherent and biofilm cultures showed decreased antibiotic susceptibility when compared with the planktonic bacteria. Planktonic bacteria were completely suscep-
tible at all concentrations. Vancomycin at twice the MBC had no significant effect on the 24 h adherent or 7 d biofilm cultures. Exposure to 10 × MBC produced no reduction in viable numbers of the 24 h adherent culture, but a 40% reduction in viable numbers of the 7 d biofilm.

**Tetracycline.** At all three antibiotic concentrations, the adherent and biofilm cultures showed decreased antibiotic susceptibility when compared with planktonic bacteria. Planktonic bacteria were completely susceptible at all antibiotic concentrations. The 24 h adherent and 7 d biofilm bacteria showed no reduction in viable numbers at twice the MBC after 24 h or 10 × MBC after 2 h. After 24 h, exposure to 10 × MBC killed 98.5% of adherent bacteria whereas the 7 d biofilm showed no reduction in viability.

**Rifampicin.** At the antibiotic concentrations used, all culture types were reduced in viable numbers by > 99% within 24 h, indicating total susceptibility under each growth condition. At shorter exposure times, the adherent and biofilm bacteria were more susceptible than the planktonic bacteria.

**Fusidic acid.** At the antibiotic concentrations used, all three culture types were completely susceptible after 24 h exposure. Adherent and biofilm cultures showed less susceptibility at shorter exposure times; data for 6 h exposure are shown in Table 2.

In the case of the antibiotics to which 7 d biofilm cultures were not susceptible at 10 × MBC (tetracycline, vancomycin and benzylpenicillin), tests were also performed using 20 × MBC. At this concentration, each of the three antibiotics killed 99% of the bacteria in all culture types within 24 h (data not shown).

Work by Pascual et al. (1993) has suggested that some catheter materials may inactivate antibiotics. To ensure that this was not occurring, organisms growing in suspension were tested in the presence of catheter material. This showed that the presence of the catheter

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**Table 2.** Effect of exposure of *S. aureus* NCTC 8325-4 to antibiotics in terms of percentage kill

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concen (× MBC)</th>
<th>Time of exposure to antibiotic (h)</th>
<th>Percentage kill in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Planktonic culture</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>2</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1</td>
<td>2</td>
<td>6-0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24</td>
<td>&gt; 99.9</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>2</td>
<td>6</td>
<td>99-0</td>
</tr>
</tbody>
</table>
Variation in colony morphology

Small (<0.2 mm) non-pigmented colonies were frequently observed (Fig. 3) among the survivors following exposure to any of the five antibiotics. They were rarely found after treatment with MBC or 2 × MBC but their frequency increased with increasing antibiotic concentrations and they were seen with equal frequency in adherent and planktonic cultures. Small colonies were also seen in the absence of antibiotic, but only in adherent cultures. Further tests could not be performed on the small colonies as they reverted to normal colony morphology with continued incubation on solid agar media.

Properties of surviving bacteria

Colonies of surviving bacteria from each antibiotic treatment were picked and cultured. In the case of vancomycin, benzylpenicillin and tetracycline, MIC and MBC characteristics were unaltered. In addition, when used to reseed fresh catheter discs which were then exposed to antibiotics, results were identical to those for the parent strain as shown previously in Table 2. This demonstrates that the surviving bacteria were not genetically resistant or a specialized subpopulation, and that their survival in adherent or biofilm culture must derive from phenotypic resistance during the attached state.

During experiments with rifampicin and fusidic acid, initial kill was sometimes followed by regrowth of bacteria, samples of which were found to be resistant when grown in suspension as well as when adherent. It was concluded that genotypic resistance had arisen in these cases and the results were discarded.

DISCUSSION

Examination of the catheter surface during the first 24 h growth (Fig. 1a) revealed that the attached culture consisted exclusively of single adherent bacteria. Bacteria were released into the supernatant during this period and the numbers released increased with time until a maximum was reached. This suggests that detachment was growth-related with one daughter cell remaining attached and the other being released, rather than the organisms randomly detaching from the surface. Scanning electron microscopy analysis showed that, with longer adherence times, single cells (Fig. 1b) became replaced by patchy areas of biofilm (Fig. 1b), suggesting that some daughter cells had remained attached. The low increase in viable numbers (an increase of about 20-fold) on the catheter discs after 7 d growth (Fig. 2) may result from a minority of new cells remaining attached and a majority being shed.

The broth in which the catheter discs were suspended was changed every 48 h to guard against nutrient depletion or accumulation of toxic metabolites. Therefore the nutrient status of the two attached cultures was similar, and any differences between them should be a result of either biofilm formation or duration of adherence.

*S. aureus*, when adherent, showed decreased susceptibility to tetracycline, vancomycin and benzylpenicillin but not to rifampicin. Fusidic acid was more active on adherent bacteria than vancomycin, tetracycline and benzylpenicillin but less active on adherent bacteria than on planktonic bacteria. In cases where adherent growth produced decreased susceptibility, it was always the MBC that was altered, the MIC remaining the same. This is therefore a case of antibiotic tolerance, which has been defined as bacteria evading only the killing action of an antibiotic with no change in the MIC (Tuomanen et al., 1986b).

Tolerant bacteria are not totally insusceptible to the killing effect of the antibiotic but lose viability more slowly than the bacteria from which they were derived (Tuomanen et al., 1986b). Virtually all studies on this phenomenon to date have been confined to cell-wall-active antibiotics. Two of the antibiotics showing elevated antibiotic tolerance in this study were cell-wall-active antibiotics, namely benzylpenicillin and vancomycin.

The commonest cause of phenotypic tolerance is slow growth (Tuomanen et al., 1986b). Non-growing bacteria evade the bactericidal activity of β-lactam antibiotics and killing rates are proportional to generation time (Tuomanen et al., 1986a), indicating that slow growth as well as non-growth affects resistance. The present study indicated that the adherent bacteria were slow-growing than the planktonic bacteria, and suggests that this may at least in part account for the tolerance observed to vancomycin and benzylpenicillin.

The third drug in this study to which attached bacteria showed a marked decrease in susceptibility was teta-
I. **protein synthesis and therefore its mode of resistance to penicillin.** It is known that tetracycline uptake requires a protonmotive force (Arima & Izaki, 1963). Genotypic resistance is associated with decreased accumulation of the antibiotic but not from planktonic cultures suggests that antibiotic susceptibility and development of SCV, therefore these two phenomena may be linked.

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**REFERENCES**


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