Comparative In Vitro Antibiotic Resistance of Surface-Colonizing Coagulase-Negative Staphylococci

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The MBCs of nafcillin, vancomycin, gentamicin and daptomycin (LY146032) were determined for three clinical isolates of coagulase-negative staphylococci grown in suspension and adherent to biomaterials. Strains studied were the slime-producing strain Staphylococcus epidermidis RP-12 (ATCC 35983), S. hyicus SE-360, and the non-slime-producing strain S. hominis SP-2 (ATCC 35982). All three strains were allowed to colonize surgical-grade disks of stainless steel, polymethylmethacrylate, and ultrahigh-molecular-weight polyethylene for 24 h, and the disks were then exposed to various concentrations of antibiotics for 24 h. Surviving adherent bacteria were mechanically dislodged from the disks and quantitated by standard broth dilution plating techniques. Biomaterial-adherent RP-12 and SE-360 yielded approximately 10 times more CFU per disk than non-slime-producing SP-2 did. For all organisms, 10 times more bacteria bound to polymethylmethacrylate disks than to the other biomaterials. In general, bacteria adherent to biomaterials exhibited greater resistance to antibiotics than the same strains in suspension did. Resistance was independent of bacterial slime-producing characteristics and was related to the biomaterial colonized.

Biomaterial-centered infections are characterized by the following features: (i) a biomaterial or damaged tissue substratum; (ii) adhesive, frequently polymicrobial bacterial colonization; (iii) persistence of infection until the substratum is removed; (iv) resistance to host defense mechanisms and antibiotic treatment; (v) specificity of materials, organisms, and location; and (vi) transformation of autochthonous or opportunistic organisms to virulent pathogens (18).

Mechanisms of antibiotic resistance have not been well characterized. A biofilm barrier effect has been proposed (4, 5, 7, 16, 30). Studies have shown that when organisms are grown in suspension they are susceptible to lower concentrations of antibiotics than when they are in surface-adherent, biofilm-enclosed populations (4, 5, 16, 20, 24). The selection of therapeutic antibiotics is usually based on standard suspension culture MIC and MBC studies. In this study, antibiotic susceptibilities of organisms grown in suspension and on biomaterials were examined.

The ability of antibiotics to kill coagulase-negative staphylococci is clinically important, since studies have shown that staphylococci are major colonizers of surgical biomaterials, including heart valves, intravascular catheters, and orthopedic appliances (1a, 6). Prosthetic infections are generally resistant to antibiotic therapy and frequently require removal of the implant before eradication of the infection is possible (12-19).

Both coagulase-positive and coagulase-negative staphylococci have been reported as causes of biomaterial-centered infections, with a tendency for coagulase-negative species to be associated with polymer-sited infections (2, 12, 15, 26). In vitro studies have indicated preferential colonization of polymer surfaces by coagulase-negative staphylococci and of metal surfaces by coagulase-positive staphylococci, although both metal and polymer surfaces are readily colonized by both coagulase-negative and coagulase-positive species (15, 16).

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MATERIALS AND METHODS

Organisms. Two strains of coagulase-negative staphylococci isolated during intravascular catheter-associated sepsis (Staphylococcus epidermidis RP-12 [ATCC 35983] and S. hominis SP-2 [ATCC 35982]) and the mouse-virulent strain S. hyicus SE-360, also a clinical isolate, were used in this study (2, 21). RP-12 is an adherent slime producer, whereas strain SP-2 has been reported to be nonadherent to smooth, inert surfaces and is not a slime producer (2). SE-360 has not been characterized for either slime production or adhesive properties, but in our laboratory it stains positive with Alcian blue, indicating slime production. All strains were initially grown from lyophilized stock in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C and maintained on brain heart infusion agar (Carr Scarborough, Decatur, Ga.) at 4°C. The three strains were also grown in cation-supplemented Mueller-Hinton broth (CSMHB) (BBL Microbiology Systems, Cockeysville, Md.) and stained with Alcian blue 8GX (Sigma Chemical Co., St. Louis, Mo.) to detect slime production (2).

Antimicrobial agents and media. Standard reference preparations of vancomycin and daptomycin (LY146032) were obtained from Eli Lilly & Co., Indianapolis, Ind.; gentamicin was obtained from Pfizer Inc., New York, N.Y.; and nafcillin was obtained from Bristol-Meyers Co., Syracuse, N.Y. Stock solutions were prepared with sterile distilled water and further diluted with CSMHB as recommended by the National Committee for Clinical Laboratory Standards (22) and as required by daptomycin for maximum inhibition of bacterial peptidoglycan synthesis (8, 9).

Daptomycin is a cyclic lipopeptide antibiotic that was originally synthesized by N-decanoyl acylation of the terminal amino group of a deacylated cyclic polypeptide antibiotic, A21978C1, derived from Streptomyces roseosporus (M. Debono, M. Barnhart, C. B. Carrell, J. A. Hoffman, and R. L. Hamill, Program Abstr. 20th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 68, 1980). It inhibits cell wall biosynthesis by blocking the incorporation of alanine (8).

**Biomaterials.** Disks (thickness, 1.5 mm) of surgical-grade stainless steel (SS) and ultrahigh molecular-weight polyethylene (UHMWPE) were cut from 7-mm-diameter rod stock with a lathe, lightly polished, and passivated with HNO₃ prior to use (Howmedica, Inc., Rutherford, N.J.). Bone cement (polymethylmethacrylate [PMMA]) components were obtained from Howmedica, combined as recommended by the manufacturer, and packed into a 7-mm (inner diameter) polypropylene cylinder. The polymerized cement was excised from the cylinder, cut into 1.5-mm cross-sections with a lathe, and soaked overnight in distilled water. SS, UHMWPE, and PMMA disks were cleaned in 500-ml volumes of 8 M urea (Sigma) at room temperature for 20 h, 5 M CaCl₂ (Sigma) at 4°C for 1 h, 5% nitric acid (Fisher Scientific Co., Norcross, Ga.) for 1 h, and two changes of sterile distilled water for 1 h each. Disks were sterilized by a 24-h exposure to ethylene oxide.

**MBCs of antibiotics for adherent bacteria.** Bacterial strains were grown in CSMHB for 6 h at 37°C from brain heart infusion slant subcultures to obtain early- to mid-log-phase organisms. The bacteria were then diluted with CSMHB against the 0.5 McFarland turbidity standard to approximately 10⁵ CFU/ml and transferred to sterile six-well plates. Biomaterial disks were placed into the bacterial suspension and incubated at 37°C on a platform rocker (Belloco Biotechnology, Vineland, N.J.). After 24 h, the disks were removed with sterile forceps, and nonadherent bacteria were removed by vigorous agitation of the disks in two 5-ml changes of isotonic phosphate-buffered saline (pH 7.2). Each disk was transferred to 1 ml of each of a series of duplicate log₂ dilution steps of antibiotic (range, 0.125 to 256 μg/ml) in 26-well plates maintained at 37°C on a platform rocker. Additional colonized disks were placed in drug-free CSMHB (controls). After 24 h of incubation, the disks were removed and agitated through two 5-ml changes of phosphate-buffered saline to remove nonadherent bacteria. The disks were placed in 10 ml of phosphate-buffered saline, sonicated for 5.5 min in a low-output cleaning sonicator, and mechanically vortexed for 30 s. Supernatants were serially diluted with phosphate-buffered saline in 10-fold steps to 10⁻⁶, and 100 μl from each tube was spread onto Columbia blood agar. Bacterial colonies were counted after 24 h of incubation at 37°C. The MBC was defined as the lowest drug concentration that resulted in at least a 99.9% reduction in the number of CFU per disk relative to control levels; no higher drug concentrations yielded less than a 99.9% kill (27). Disks with adherent bacteria were examined under an electron microscope before and after vortexing and sonication to ensure complete removal of the bacteria from the disks.

**MBCs of antibiotics for suspended bacteria.** The MBC tests were performed in duplicate on suspended bacteria by modification of the MIC microdilution method described previously (22). Bacterial inocula were standardized against the 0.5 McFarland turbidity standard and diluted with CSMHB to produce a final concentration of approximately 10⁶ CFU per well in each of a series of log₂ antibiotic dilutions. MBCs were determined after 24 h of incubation at 37°C by mixing the contents of wells showing no growth and streaking 10 μl over a quadrant of a Columbia blood agar plate (25, 27, 29). The plates were read after 24 h of incubation at 37°C. Repeat MBC tests were also performed by using initial inocula of 10³, 10⁴, and 10⁵ to rule out an inoculum effect that may be present when MBCs determined for suspension organisms and biomaterial-adherent organisms are compared.

**RESULTS**

**Bacterial colonization on SS, UHMWPE, and PMMA.** A comparison of the colonization of biomaterials by the three strains of coagulase-negative staphylococci is presented in Table 1. The non-slime-producing SP-2 strain exhibited significantly fewer CFU per disk than did the slime-producing RP-12 strain and SE-360 (P < 0.05), indicating a reduced capacity of SP-2 to adhere to the disks. In all instances, SP-2 yielded about 90% fewer organisms per disk than did RP-12 and SE-360, with adherence capacities of statistically equal value. All strains exhibited greater colonization of PMMA than either SS or UHMWPE.

**Slime production.** Selective staining with Alcian blue for mucopolysaccharide slime showed RP-12 to be the only significant slime producer of the three strains tested. Although SE-360 stains lightly with Alcian blue, it cannot be classified as a significant slime producer.

**Comparative in vitro activities of nafcillin, vancomycin, gentamicin, and daptomycin against three strains of coagulase-negative staphylococci.** The slime-producing strain S. epidermidis RP-12 was most susceptible to nafcillin (MBC, 0.5 μg/ml) and daptomycin (2.0 μg/ml) and relatively resistant to vancomycin (8 μg/ml) and gentamicin (256 μg/ml).

<table>
<thead>
<tr>
<th>Strain and antibiotic</th>
<th>MBC(*) (μg/ml) for suspended bacteria</th>
<th>MBC(*) (μg/ml) for bacteria adherent to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>UHMWPE</td>
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<tr>
<td><strong>RP-12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Nafcillin</td>
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<td>128</td>
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<tr>
<td>Vancomycin</td>
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<td>32</td>
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<tr>
<td>Gentamicin</td>
<td>256</td>
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<tr>
<td><strong>SE-360</strong></td>
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<tr>
<td>Daptomycin</td>
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<td>Gentamicin</td>
<td>2</td>
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<tr>
<td><strong>SP-2</strong></td>
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<tr>
<td>Daptomycin</td>
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<td>1</td>
</tr>
<tr>
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<tr>
<td>Vancomycin</td>
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<td>Gentamicin</td>
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* Values are representative of the MBC obtained from two simultaneously treated specimens. Organisms were allowed to adhere to disks for 24 h prior to 24 h of antibiotic exposure.
when grown in suspension (Table 2). This organism showed increased resistance to daptomycin when bound to UHMWPE and PMMA. RP-12 showed increased resistance to nafcillin when the organism was in its biomaterial-adherent state, but this may be related to an inoculum effect (Table 3).

The minimally slime-producing strain SE-360 \( (S. \) \text{lycos} \) grown in suspension was susceptible to nafcillin (MBC, 0.5 \( \mu \text{g/ml} \)), daptomycin (2 \( \mu \text{g/ml} \)), and gentamicin (2 \( \mu \text{g/ml} \)) but resistant to vancomycin (16 \( \mu \text{g/ml} \)). Once adherent to biomaterial, SE-360 displayed increased resistance to all three antibiotics to which it had previously been susceptible. This was particularly evident for adherence to PMMA. SE-360 did not show an inoculum effect toward any of the antibiotics tested (Table 3).

The non-slime-producing coagulase-negative strain \( S. \) \text{hominis} SP-2 was susceptible to nafcillin (MBC, 0.25 \( \mu \text{g/ml} \)) and daptomycin (1.0 \( \mu \text{g/ml} \)) and relatively resistant to vancomycin (8 \( \mu \text{g/ml} \)) and gentamicin (16 \( \mu \text{g/ml} \)) when grown in suspension. Biomaterial-adherent strain SP-2 exhibited increased resistance to daptomycin when the organism was bound to UHMWPE and PMMA but not to SS. When nafcillin was the challenging antibiotic, resistance was increased on SS and PMMA but not on UHMWPE.

**DISCUSSION**

The data presented on the susceptibility of biomaterial-adherent coagulase-negative staphylococci to antibiotics compared with the susceptibility of organisms grown in suspension revealed that biomaterial-adherent bacteria had an increased resistance to antibiotics. This is consistent with previous reports in which essentially one antibiotic and one substratum were examined (4, 5, 16, 23, 24, 28; W. W. Nichols, M. J. Evans, M. P. E. Slack, and H. L. Walmsley, J. Gen. Microbiol., in press).

In this study, we compared several antibiotics and several biomaterials and noted that antibiotic resistance is apparently unrelated to the production of bacterial exopolysaccharides. This conclusion is exemplified by the susceptibility of the slime-producing strain RP-12 and the non-slime-producing strain SP-2 to daptomycin when the organisms were adherent to SS, compared with the resistance of the slime-producing strain SE-360 to daptomycin when the organism was adherent to SS.

The level of resistance exhibited by biomaterial-adherent strains RP-12 and SE-360 to nafcillin was unpredictable on the basis of the MBCs of nafcillin for these strains when tested in suspension cultures. More importantly, the antibiotic resistance of MBC for all strains adherent to SS, UHMWPE, and PMMA could not be predicted by MBC determinations for the same bacteria growing in suspension.

Biomaterial correlation was displayed by SP-2 susceptibility to nafcillin when the organism was adherent to UHMWPE and its resistance to nafcillin when it was adherent to SS or PMMA. RP-12 also exhibited biomaterial effects, being relatively susceptible to daptomycin when the organism was bound to SS, compared with its resistance when adherent to UHMWPE or PMMA. The most consistent findings in these studies were the antibiotic resistance of all organisms examined when adherent to PMMA and the relative biomaterial specificity of resistance.

The data on bacterial binding to biomaterials revealed several other interesting findings. First, exopolysaccharide-producing organisms bound more bacteria per unit surface area than the non-slime-producing organisms did, suggesting a role for slime in quantitative bacterial adherence. Second, an increased number of organisms bound to PMMA per unit area than to SS or UHMWPE, indicating a possible biomaterial specificity for quantitative bacterial adherence of the biomaterial.

It has been suggested that the polyanionic nature of the mucopolysaccharide slime may form a diffusion barrier and inhibit the access of antibiotics to the cell wall (3–5, 11–13, 17, 21, 28). The facts that the slime-producing strain RP-12 did not exhibit greater resistance than the non-slime-producing strains SE-360 or SP-2 and that the non-slime-producing adherent strain SP-2 exhibited a substantial resistance to all antibiotics, particularly when the organism was adherent to PMMA, suggest that the role of exopolysaccharide slime in the resistance of \( S. \) \text{epidermidis} to antibiotics is questionable.

The surfaces of SS, PMMA, and UHMWPE provide a unique environmental niche to which coagulase-negative staphylococcal strains adhere and colonize. The colonization of these biomaterial surfaces with bacteria and the interaction between them somehow abrogate the action of antibiotics. Although excess exopolysaccharide slime production may interfere with antibiotic penetration, it is unlikely that this is the mechanism by which adherent organisms withstand high levels of antibiotics. Recent studies by Nichols (23; Nichols et al., in press) suggest that extracapsular polysaccharides do not present a significant diffusion barrier to antibiotics and that although binding to exopolysaccharides does occur, a sufficient amount of antibiotic does reach the cells and surfaces over a course of treatment.

Biomaterial surface or nutrient conditions in microcolonies at the surface may also increase or decrease the metabolic rate (1, 10, 12; Nichols et al., in press). Another explanation is that bacteria within nutrient-starved portions of surface colonies have decreased metabolic rates, resulting in an increased resistance to antibiotics (1, 10, 23; Nichols et al., in press).

In summary, bacteria on surfaces or within microcolonies are probably physiologically different from organisms in suspension, and therefore their response to antibiotics may be altered, depending on the specific chemistry of the cell and the mechanism of action of the antibiotic.

The findings presented here suggest that the degree of colonization and antibiotic resistance are species related and substratum (biomaterial) directed and, to a degree, may be altered by substratum-induced phenotypic changes rather than by a barrier effect of exopolysaccharides.
ACKNOWLEDGMENTS

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LITERATURE CITED


