Effects of Subinhibitory Concentrations of Vancomycin or Cefamandole on Biofilm Production by Coagulase-Negative Staphylococci

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The density of the biofilm layer produced on a plastic surface by 23 clinical isolates and 1 reference strain of slime-positive, coagulase-negative staphylococci was measured following growth in subinhibitory concentrations (sub-MICs) of cefamandole or vancomycin ranging from 2 to 0.008 μg/ml. All strains were susceptible to ≤2 μg of each agent per ml. The mean biofilm density produced by each strain was calculated from a total of eight determinations at each sub-MIC and was compared with the mean biofilm density of a drug-free control after correcting for differences in growth. The results showed that the density of the biofilm layer produced by 10 (42%) of 24 strains and 13 (54%) of 24 strains was significantly increased (P < 0.006) at one or more sub-MICs of cefamandole or vancomycin, respectively. In contrast, the density of the biofilm produced by 9 (38%) of 24 and 2 (8%) of 24 strains was significantly reduced at one or more sub-MICs of cefamandole and vancomycin, respectively, and the biofilm density of 7 of these strains was decreased only when the sub-MIC was one-half the MIC. The biofilm density of six strains (five versus cefamandole and one versus vancomycin) was both enhanced and reduced by different sub-MICs of the same agent. None of the strains produced a detectable biofilm at or above the MIC for the strain. These data indicate that antimicrobial agents such as cefamandole or vancomycin could potentially enhance the biofilm matrix produced by certain slime-positive, coagulase-negative staphylococci on the surface of a biomedical implant if concentrations of these agents fall below the MIC for the infecting strain.

Slime-producing (S+), coagulase-negative staphylococci (CONS), especially Staphylococcus epidermidis, frequently cause foreign-body infections of implantable biomedical devices including cardiac valves (8), intravascular or peritoneal catheters (2, 5, 13, 21), and cerebrospinal fluid shunts (22). Although the pathogenesis of implant-associated infections is not entirely clear, it is presumed that organisms infect a device through one of three routes: direct surgical contamination, transient bacteremia, or extra- or intraluminal migration along transcutaneous devices (1). Infection and subsequent colonization of a biomedical implant can be divided chronologically into several phases consisting of adherence, growth, and slime production (9, 19). The first phase, bacterial adherence, appears to be controlled in vitro by hydrophobic or electrostatic surface interactions between CONS and the substratum (12). Host tissue or plasma proteins deposited on the surfaces of medical implants may be important determinants of bacterial adhesion in vivo, although the addition of plasma or the plasma protein fibronectin to hydrophobic surfaces reduces the adherence of CONS in vitro (12; W. M. Dunne, P. L. Havens, and F. T. Counter, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 640, 1989).

The matrix of bacteria, slime, and exogenous factors (collectively termed a biofilm layer) which envelops the surface of a colonized implant anchors S+ CONS firmly to the surface of the device (9, 19) and protects adherent bacteria from host defenses (14). Once a bacterial biofilm has formed on the surface of an implant, therapeutic options are often limited to long-term administration of antimicrobial therapy or removal of the infected device or both (8, 19). Antimicrobial therapy alone, even with agents demonstrating in vitro efficacy against the offending strain, is seldom an effective means of sterilizing the surface of an infected implant (17).

Most strains of S+ CONS recovered from infected implants are resistant to a broad spectrum of antimicrobial agents, including beta-lactam antibiotics, macrolides, sulfonamides, trimethoprim, chloramphenicol, and the aminoglycosides (2, 7, 8, 22). With rare exception, most isolates remain susceptible to rifampin and vancomycin. The in vitro activities of narrow- and expanded-spectrum cephalosporins such as cephalothin and cefamandole are variable. Cefamandole has demonstrated good inhibitory activity in vitro against both methicillin-susceptible and methicillin-resistant CONS (10).

Sterilization of cerebrospinal fluid shunts infected by CONS with vancomycin alone has been reported (22), but the therapeutic success rate was significantly higher when infecting strains were slime negative or when vancomycin therapy was used in conjunction with partial or complete revision of the shunt. Recent data reported by Frongillo et al. (11) indicate that cefamandole may be efficacious in the treatment of serious infections caused by CONS (methicillin susceptible or resistant), including foreign-body infections. However, the slime phenotype of recovered isolates was not determined in this study.

The inconsistent success of antimicrobial therapy for prosthetic-implant infections may reflect the poor penetration of antibiotics through a biofilm matrix with subsequent exposure of adherent organisms to subinhibitory concentrations (sub-MICs) of potentially effective agents. Previous studies have shown that sub-MICs of certain antimicrobial agents influence (both positively and negatively) the adherence of a variety of microorganisms, including CONS (16, 20). Because of the potential utility of either cefamandole or
vancomycin for prophylaxis against and therapy of biomedical-implant infections caused by S+ CONS, this investigation examined the effects of sub-MICs of each agent on the biofilms produced by 24 clinical isolates of S+ CONS in vitro. (This work was presented in part at the 89th meeting of the American Society for Microbiology, New Orleans, La., 14 to 18 May 1989.)

MATERIALS AND METHODS

Bacterial strains. A total of 23 strains of S+ CONS were collected from clinical specimens submitted to the microbiology laboratories of Children’s Hospital of Wisconsin and the Zablocki Veteran’s Administration Hospital, both in Milwaukee, Wis. Slime production was determined by the qualitative tube assay of Christensen et al. (3). The collection consisted of 7 catheter isolates, 14 blood isolates, and 2 cerebrospinal fluid (shunt) isolates. All clinical S+ CONS were identified as S. epidermidis by the API Staph-Ident system (Analytab Products, Plainview, N.Y.). An additional strain of S+ CONS (S. epidermidis ATCC 35984) previously characterized by Christensen et al. (3) was included as a reference isolate.

Assay of biofilm density. All measurements of biofilm density were performed by using a modified version of the microwell assay described by Christensen et al. (4). Sterile, flat-bottomed 96-well polystyrene tissue culture plates (GIBCO Laboratories, Grand Island, N.Y.) were used as a substrate for biofilm production. The plates were prepared as follows. Columns 1 and 12 contained 200 μl of tryptic soy broth (TSB; GIBCO) without antibiotics and served as a negative control. Column 2 contained 100 μl of TSB without antibiotics and was used as an antibiotic-free growth control. Columns 3 through 11 contained 100 μl of serial twofold dilutions of either vancomycin or cefamandole ranging from 4 to 0.015 μg/ml. Prepared plates were stored at −80°C and thawed immediately prior to use. To perform the assay, individual strains were cultured in 0.5 ml of brain heart infusion broth for 2 h at 37°C. Ten microliters of the broth was transferred into 10 ml of TSB and mixed thoroughly. A portion (100 μl) of the resulting inoculum (approximately 5 × 10^7 organisms per ml) was delivered to all eight wells in columns 2 through 11 for a final volume of 200 μl per well and sub-MICs ranging from 2 to 0.008 μg/ml.

Following an 18-h incubation at 37°C, the turbidity of bacterial growth in each well (growth index) was measured at 570 nm by using an MR 580 microELISA AutoReader (Dynatech Laboratories, Inc., Alexandria, Va.), with columns 1 and 12 (uninoculated TSB) used to blank the instrument. The bacterial growth was then decanted from the plate, and each well was washed four times with Hank’s balanced salt solution (GIBCO) to remove nonadherent bacteria. The remaining biofilm was fixed for 10 min in absolute methanol, dried at 45°C, and measured at 570 nm. Measurements of the biofilm layer were divided by corresponding growth index values, to correct for differences in biofilm density related to variations in the bacterial density of individual wells and to compensate for partial growth inhibition caused by sub-MICs of cefamandole or vancomycin at lower dilutions. Correcting for growth with turbidity measurements was possible because the relationship between cell count and A_570 was linear and nearly proportional (slope = 0.91, r = 0.99) over the range of measured turbidity (0.008 to 0.6 A_570). A mean biofilm density was then calculated for each sub-MIC from the eight corrected values and was compared with the mean biofilm density of an internal drug-free control by using the two-tailed Student’s t test corrected for multiple comparisons by the method of Bonferroni (15). By using this method of analysis and ATCC 35984 as a control strain, only one discrepant result was observed with vancomycin, but none were observed with cefamandole when the assay was performed on three consecutive days (data not shown). In addition, there were no significant differences in the mean biofilm density between columns of a single plate when the assay was performed in the absence of antimicrobial agents. Following the measurement of biofilm density, all microdilution plates were stained by the method described by Christensen et al. (4). However, under the conditions described here, the absorbance of stained biofilms often exceeded the threshold of the instrument (1.5 A) and could not be used for comparison.

Susceptibility testing. The MICs of cefamandole and vancomycin for the test strains of CONS were determined by broth microdilution testing according to published guidelines (18), with cation-supplemented Mueller-Hinton broth. Previous studies had shown that 19 of the 24 S+ CONS were resistant to oxacillin (6).

RESULTS

Cefamandole. All strains of S+ CONS were susceptible to 2 μg or less of cefamandole per ml by microdilution well susceptibility testing. All MICs obtained with supplemented Mueller-Hinton broth were within one doubling dilution of those observed with TSB. None of the S+ CONS produced a detectable biofilm layer at or above the MIC for individual strains. The densities of the biofilm produced by 10 (42%) of 24 strains were significantly increased (P < 0.006) at one or more sub-MICs of cefamandole. The greatest increase in biofilm density produced by a single strain (411% of control) corresponded to a cefamandole concentration of 0.06 μg/ml. The biofilm produced by one strain of S+ CONS was significantly enhanced at all sub-MICs of cefamandole tested. Biofilm enhancement was detected in the greatest number of test strains (seven) at cefamandole concentrations of 0.12 and 0.06 μg/ml (Fig. 1).

The densities of biofilm formed by 9 (38%) of 24 S+ CONS were significantly reduced at one or more sub-MICs of cefamandole. The biofilm layer produced by two strains was significantly decreased at all sub-MICs of cefamandole tested. Cefamandole at a concentration of 0.5 μg/ml caused biofilm reduction in the greatest number of strains (five; Fig. 1). The densities of biofilm produced by seven S+ CONS were significantly reduced only when the sub-MIC of cefamandole equalled one-half the MIC for the strain.

FIG. 1. Influence of subinhibitory concentrations of cefamandole on the density of biofilm produced by 24 strains of S+ CONS. The histogram shows the frequency and distribution of strains in which biofilm production was significantly affected as a function of decreasing concentrations of cefamandole.

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mycin can enhance and repress the formation of a bacterial biofilm produced by select strains of S+CONS in vitro. At least one sub-MIC of either antimicrobial agent caused a significant increase in the density of the biofilm layer produced by 17 (71%) of 24 test strains. Vancomycin and cefamandole were comparable in that they stimulated increased biofilm production by 12 and 9 strains, respectively. Compared with sub-MICS of cefamandole, however, sub-MICs of vancomycin were less likely to suppress biofilm formation. The biofilm layers produced by 9 of 24 strains were significantly reduced by sub-MICs of cefamandole, compared with only 2 of 24 strains treated with vancomycin. The densities of the biofilm layers formed by six strains (five treated with cefamandole and one treated with vancomycin) were alternately increased and decreased at different sub-MICs of the same agent. Interestingly, all reductions in biofilm density occurred when the sub-MIC was equal to one-half the MIC for the strain. At that concentration, a significant reduction in growth (P < 0.001 by analysis of variance) compared with that of the drug-free growth control was also noted for each similarly affected strain. Strains in which the biofilm density was unaffected did not show a similar reduction in growth index at one-half the MIC. The growth indices of strains demonstrating a significant increase in biofilm density were not significantly greater than those for controls at any sub-MIC. Despite efforts to compensate for differences in bacterial density, it appears that biofilm reduction is likely related to growth inhibition, while increased biofilm density is independent of cell density.

Prior to this study, two independent groups reported on the effects of sub-MICS of antimicrobial agents, including vancomycin, on biofilm formation by CONS (20; M. Pfaller, D. Davenport, M. Bale, M. Barrett, F. Koontz, and M. Massanari, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, L-43, p. 419). Similar to the results described here, the study of Pfaller et al. demonstrated a 14% reduction in the slime layer (biofilm) produced by nine strains of S+CONS at concentrations of vancomycin equal to one-half the MIC. No increase in biofilm production was observed at higher dilutions of vancomycin. Schadow et al. (20) found that sub-MICs of vancomycin had no effect on the slime layer produced by three S+CONS and one slime-negative strain of CONS. Neither of the previous studies corrected measurements of residual biofilm layers for differences in bacterial cell density. In the present study, staining of biofilms (depending upon the strain of S+CONS) often produced optical measurements which exceeded the capacity of the microELISA reader and which were not useful for comparison. Other variables among the three studies included length of incubation, number of strains examined, and number of sub-MICs included for comparison. Despite these differences, all three reports concluded that certain antimicrobial agents could significantly reduce the biofilm layer produced by S+CONS at subinhibitory concentrations, but none demonstrated complete suppression of biofilm formation below the MIC for the organism.

It would be difficult to predict the in vivo response of S+CONS causing foreign-body infections to subinhibitory concentrations of various antimicrobial agents on the basis of the results of the in vitro model described here and in the two previous studies. None of the investigations incorporated serum or plasma proteins, which may be important determinants of bacterial adherence and matrix formation, into the assay despite evidence suggesting that plasma proteins, including fibronectin, may actually decrease the primary adherence of S+CONS to plastic (12; W. M. Dunne,

The densities of biofilm formed by five strains were both increased and decreased at different sub-MICs of cefamandole. With each strain, however, the reduction occurred only when the sub-MIC was equal to one-half the MIC for the strain, while increased biofilm production was observed at lower concentrations of cefamandole.

The densities of biofilm produced by 10 strains were unaffected by sub-MICs of cefamandole. No correlation between oxacillin resistance and the effect of sub-MICs of cefamandole on biofilm production could be discerned.

**Vancomycin.** All strains of S+CONS were susceptible to ≤2 μg of vancomycin per ml by broth microdilution susceptibility testing, and as with cefamandole, the MICs recorded for TSB were within one doubling dilution of those obtained with supplemented Mueller-Hinton agar. Also as with cefamandole, none of the test strain S+CONS produced a measurable biofilm layer at or above the MIC of vancomycin.

The densities of the biofilm layers produced by 13 (54%) of 24 S+CONS were significantly greater than that of the drug-free control at one or more sub-MICs of vancomycin. The greatest increase was recorded for two S+CONS at 0.5 μg/ml, at which concentration mean biofilm densities exceeded control values by 723% and 867%, respectively. The biofilm produced by three strains was significantly increased at all sub-MICs of vancomycin. Vancomycin promoted enhanced biofilm synthesis by nine S+CONS at a concentration of 0.03 μg/ml (Fig. 2).

Vancomycin caused a significant reduction in the density of biofilm formed by two strains at one or more sub-MICs. However, no single sub-MIC of vancomycin suppressed biofilm density in more than one test strain of S+CONS (Fig. 2).

Only one strain showed both a significant decrease and an increase in biofilm density at different sub-MICs of vancomycin. Similar to the effects of cefamandole, the reduction in biofilm density corresponded to a vancomycin concentration equal to one-half the MIC for the strain, with increased biofilm synthesis being measured as the concentration of vancomycin was diluted.

The densities of biofilm layers produced by 10 strains were unaffected by sub-MICs of vancomycin. No relationship between the effects of vancomycin on biofilm density and oxacillin resistance was apparent.

**DISCUSSION**

The data presented in this study have shown that sub-MICs of the antimicrobial agents cefamandole and vanco-

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P. L. Havens, and F. T. Counter, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 640, 1989). In addition, each study utilized static concentrations of antimicrobial agents which would not occur in vivo, although the number of dilutions examined in this report spanned a wide range of subinhibitory concentrations. Despite these deficits, several conclusions can be drawn from the combined data. First, none of the test strains of S+CONS in this or in previous studies produced a measurable biofilm layer at or above the MIC for the strain. Secondly, antimicrobial agents such as vancomycin or cefamandole could potentially stimulate the formation of a biofilm layer by S+CONS on hydrophobic surfaces when levels fall below inhibitory concentrations. Collectively, these findings stress the importance of maintaining or exceeding MICs of prophylactic or therapeutic antimicrobial agents at the site of a biomedical implant to prevent or eradicate infection by strains of S+CONS.

LITERATURE CITED


