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 15 (60%) 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 24 (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 substrate (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 cephalexin 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 biomed-
ical-implant infections caused by S+ CONS, this investiga-
tion examined the effects of sub-MICs of each agent on the
biofilms produced by 24 clinical isolates of S+ CONS in vitro.
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American Society for Microbiology, New Orleans, La., 14 to
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MATERIALS AND METHODS

Bacterial strains. A total of 23 strains of S+ CONS were
collected from clinical specimens submitted to the microbi-
ology 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 collect-
cion 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-Iden-
ty system (Analytab Products, Plainview, N.Y.). An addi-
tional 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
substratum 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
were 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 ×
10⁵ 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 col-
umns 1 and 12 (uninoculated TSB) used to blank the instru-
ment. The bacterial growth was then decanted from the
plate, and each well was washed four times with Hanks
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 corre-
sponding 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 vancomy-
cin at lower dilutions. Correcting for growth with turbidity
measurements was possible because the relationship be-
tween cell count and A₅₇₀ was linear and nearly proportional
(slope = 0.91, r = 0.99) over the range of measured turbidity
(0.008 to 0.6 A₅₇₀). A mean biofilm density was then calcu-
lated 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 Bon-
feronni (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 signif-
icant 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 measure-
ment 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 instru-
ment (1.5 A) and could not be used for comparison.

Susceptibility testing. The MICs of cefamandole and van-
comycin for the test strains of CONS were determined by
broth microdilution testing according to published guidelines
(18), with cation-supplemented Mueller-Hinton broth. Pre-
vious 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 signif-
icantly 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 cefa-
mandole equalled one-half the MIC for the strain.
FIG. 2. Influence of subinhibitory concentrations of vancomycin 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 altered as a function of decreasing concentrations of vancomycin.

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 vancomycin 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. Pfaffler, 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 Pfaffler 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 MICs. 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,
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