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Effect of Vancomycin Hydrochloride on *Staphylococcus epidermidis* Biofilm Associated with Silicone Elastomer

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Peritonitis is a major complication of continuous ambulatory peritoneal dialysis. Relapsing peritonitis after the cessation of antimicrobial therapy is frequently reported and often involves *Staphylococcus epidermidis*. To investigate the potential role of catheter-associated biofilm in the pathogenesis of relapsing peritonitis, we describe an in vitro model permitting the development of an *S. epidermidis* biofilm on silicone elastomer biomaterial. This model has been used to investigate the ability of vancomycin hydrochloride to kill biofilm-encased organisms by using an antibiotic regimen typical of peritonitis therapy. No significant differences were seen between vancomycin-exposed and control groups in biofilm viable and total cell counts after 10 days. Vancomycin-exposed silicone-associated biofilm populations decreased by only 0.5 $\log_{10}$ CFU/cm^2 over the study period. MICs and MBCs for the original *S. epidermidis* suspension were 3.125 and 6.25 μg/ml, respectively. For biofilm homogenate suspensions, MICs were 3.125 μg/ml, but MBCs were >400 μg/ml. These data indicate that the biofilm organisms associated with an indwelling peritoneal catheter may display a form of tolerance, thereby suggesting one possible mechanism behind relapsing peritonitis.

Microbial biofilms, consisting of adherent, dense populations of microorganisms and their associated exopolysaccharides, have been frequently observed on the surfaces of indwelling and implantable medical devices and prostheses upon removal from their hosts (2, 6, 8, 27, 31). The ability of various microorganisms to attach to inert surfaces and further develop into biofilms has been implicated as a significant factor in the persistent nature of foreign-body infections typically involving cerebrospinal fluid shunts, vascular grafts, pacemaker leads, and various indwelling catheters (2, 14, 26, 30). *Staphylococcus epidermidis* is often reported as a major opportunistic pathogen in foreign-body infections (2, 9, 24). Several studies have suggested that the production of exopolysaccharide or "slime" by this species of coagulase-negative staphylococcus may promote biofilm development on inert surfaces and may also function as a virulence factor (2, 6, 9, 18, 21, 23). It is well recognized that antimicrobial therapy of medical-device-associated infections often requires concurrent removal of the device for an effective cure. An explanation of this characteristic is offered by Nickel et al., who demonstrated the inability of tobramycin to kill *Pseudomonas aeruginosa* cells embedded in a biomaterial-associated biofilm at antibiotic levels of greater than 50 times the MBC for the identical strain grown in liquid suspension (29).

Peritonitis is the most common complication of continuous ambulatory peritoneal dialysis. In a recent analysis of the causative pathogens in continuous ambulatory peritoneal dialysis-associated peritonitis, Golper and Hartstein (15) confirmed the previously well-established high incidence of *S. epidermidis*, accounting for 43% of all infectious episodes in their report. In addition, the latter study reported that two-thirds of all infections were caused by the same pathogen as in the immediately preceding infection and suggested that staphylococcal infections may be particularly prone to relapse when treated with short and therefore presumably ineffective courses of antibiotics. Furthermore, Dasgupta et al. have confirmed the ubiquitous presence of microbial biofilms on peritoneal catheters retrieved from continuous ambulatory peritoneal dialysis patients (8).

To study the response of biofilm-encased bacteria to antibiotics in an in vitro system simulating the therapeutic conditions of continuous ambulatory peritoneal dialysis, we developed a model permitting the development of a staphylococcal biofilm on peritoneal catheter biomaterial. The purpose of this investigation was to evaluate the activity of vancomycin against an *S. epidermidis* silicone elastomer-associated biofilm. Vancomycin is frequently selected for the treatment of gram-positive bacterial peritonitis in continuous ambulatory peritoneal dialysis patients (3, 19).

MATERIALS AND METHODS

Bacterial strain. The *S. epidermidis* strain used in this study was a clinical isolate from a continuous ambulatory peritoneal dialysis patient presenting with peritonitis (isolate 8-14B from Timothy E. West, School of Medicine, University of Buffalo, State University of New York, Buffalo) and was slime positive in the safranin test (6). This isolate was maintained between experiments on Bacto Nutrient Agar slants (Difco Laboratories, Detroit, Mich.) at 5°C.

Synthetic dialysis effluent medium. A synthetic dialysis effluent containing the following ingredients was formulated from the mean chemical analysis value of eight patient-retrieved dialysis effluents: $CaCl_2 \cdot 2H_2O$ (133 μg/ml), $MgCl_2 \cdot 6H_2O$ (81 μg/ml), NaCl (8 mg/ml), KH_2PO_4 (128 μg/ml), KCl (197 μg/ml), urea (1.32 mg/ml), creatinine (83 μg/ml), and glucose (3.37 mg/ml); pH, 7.25; osmolality, 300 mosmol; total carbon/total nitrogen ratio, 2:1. In addition, the formulation was supplemented with 0.4% (wt/vol) nutrient broth (28). The medium, minus calcium, magnesium, urea, and creatinine, was autoclaved for 35 min at 121°C. The remaining ingredients were filter sterilized and aseptically added to the heat-sterilized solution. The nutritional adequacy of this formula was confirmed by growth curve determination, which confirmed the ability of the synthetic medium to support the proliferation of *S. epidermidis* at a level comparable to that in normal patient dialysis effluent (25, 34, 36).

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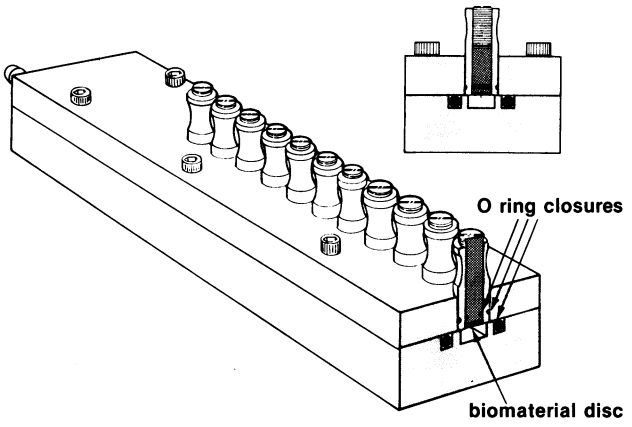

FIG. 1. Cross-section view of the biological sampling device. The design is based on that of the modified Robbins device (27).

A stock solution containing 1 mg of vancomycin hydrochloride (Eli Lilly & Co., Indianapolis, Ind.) per ml was prepared in sterile distilled water and stored at 4°C. When required, vancomycin was added to the synthetic dialysis effluent medium from the stock solution to obtain a working concentration of 25 μg/ml. Antibiotic potency was confirmed by an agar diffusion assay by using tryptic soy agar seeded with *Bacillus subtilis* ATCC 6633. Wells punched into the agar were inoculated with standard and test solutions of vancomycin hydrochloride in synthetic dialysis effluent (31).

Biofilm model. A stainless steel modification of a Robbins device (27) (Fig. 1) was used to create the bacterial biofilm. This device had a total volume of 16 ml and contained 25 removable sampling ports in which 6-mm-diameter biomaterial disks could be inserted, permitting the exposed disk surface to remain flush with the upper interior wall of the fluid flow path. This design prevented the occurrence of artifacts of cell sedimentation on the biomaterial and allowed salient fluid dynamic features to be defined. Thus, at an operating flow rate of 60 ml/h, the Reynolds number was 1.6 (nonturbulent flow), the wall shear rate was 18.08/s, and the wall shear stress was 0.27 dynes/cm^2 (27 × 10^{-3} N/cm^3).

Experimental design. Twenty-five disks of medical-grade silicone elastomer (General Electric Co., Schenectady, N.Y.) were placed in the sampling inserts and cleaned by sequentially rinsing with distilled water, 95% ethyl alcohol, and distilled water. The inserts were then placed in the sampling device and steam sterilized at 121°C for 45 min. *S. epidermidis* was inoculated from Nutrient Agar slants into sterile synthetic dialysis effluent medium and incubated for 12 h at 37°C. This suspension (5 ml) was then added to a 250-ml reservoir of synthetic dialysis effluent medium, incubated for 12 h at 37°C, and passed through the sampling device for 2 h to seed the silicone disks with *S. epidermidis*. The silicone-containing inserts were subsequently removed aseptically and transferred to a second sterile device through which sterile synthetic dialysis effluent medium was passed at a flow rate of 60 ml/h continuously for 72 h at 37°C to permit the development of a confluent biofilm. After the 2-h seeding phase and again after 72 h of continuous fluid flow, 2 to 3 disks were randomly removed from the device for viable and total cell quantitation and surface examination by scanning electron microscopy (SEM). After 72 h in the device, each remaining biofilm-coated disk was individually transferred to either a test tube containing 10 ml of sterile synthetic dialysis effluent medium plus 25 μg of vancomycin per ml or a control tube containing 10 ml of sterile synthetic dialysis effluent medium with no antibiotic. This second phase of the experiment simulated static fluid in the peritoneum and attempted to model the response of a catheter-associated biofilm to intraperitoneally instilled vancomycin. Disks were transferred daily into fresh sterile synthetic dialysis effluent medium with or without vancomycin for 10 days. Two to three disks from both test and control tubes were used for viable and total cell quantitation and SEM after 1, 4, 7, and 10 days of incubation at 37°C. In addition, the viable cell population of the synthetic dialysis effluent medium in both test and control test tubes was quantitated at each disk sampling period. The experiment was done five times.

Bacterial quantitation. Before we attempted to quantitate silicone disk-associated biofilm, nonadherent organisms and organisms present in any carry-over suspension were removed by passive diffusion by immersing each disk in 10 ml of sterile water for 15 min. This step was done three more times. Each disk was then placed in a precooled (−20°C) stainless mini blender cup (Waring Products Div., New Hartford, Conn.) containing 10 ml of frozen (−20°C) Letheen broth (Difco). Disks were then homogenized for 2 min at high speed to remove and disaggregate the biofilm. The temperature of the homogenate always remained below 35°C because of the precooling procedures described above. Control tests showed that this blending process did not affect cell viability. The blended homogenate was then inoculated, in duplicate, into impedance modules containing modified plate count broth (12) and quantitated by using an automated impedance detection system (model 123 Microbial Monitoring System; Bactomatic, Inc., Princeton, N.J.). A calibration curve correlating impedance detection time to CFU gave a correlation coefficient value of −0.97 for the bacterial strain under study.

Total bacterial counts of the homogenate were obtained by the acridine orange direct count method (29). Briefly, each homogenate was sonicated at 100 W for 2 min and then filtered through a 25-mm-diameter, 0.2-μm-pore-size polycarbonate filter (Nuclepore Corp., Pleasanton, Calif.). The filter was then stained with acridine orange and examined by epifluorescence microscopy. A total of 10 fields were counted per filter.

SEM. After passive diffusive rinsing as described above, disks were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M) for a minimum of 8 h. Disks were then dehydrated through an ethanol series (20 to 100%), followed by two changes of Freon 113 before air drying. The disks were then mounted on carbon SEM supports, sputter coated with gold, and examined on a JEOL JXA 35 scanning microprobe (JEOL, Peabody, Mass.).

Susceptibility tests. MIC and MBC tests were done on the original *S. epidermidis* culture, the 72-h effluent suspension of the sampling device, and a homogenate of the biofilm-derived bacteria present at 72 h on the disk just before antibiotic exposure. The macrobroth dilution method with cation-supplemented Mueller-Hinton broth was used with an inoculum of 10^5 CFU/ml for all tests (22). MICs were recorded after 24 h of incubation. The MBC was defined as the lowest concentration of antibiotic which produced ≧99.9% killing of the inoculum, as determined by 0.1-ml subcultures of the homogenized MIC tube contents onto soybean casein digest agar. Susceptibility tests were also done in parallel by substituting synthetic dialysis effluent medium for Mueller-Hinton broth.

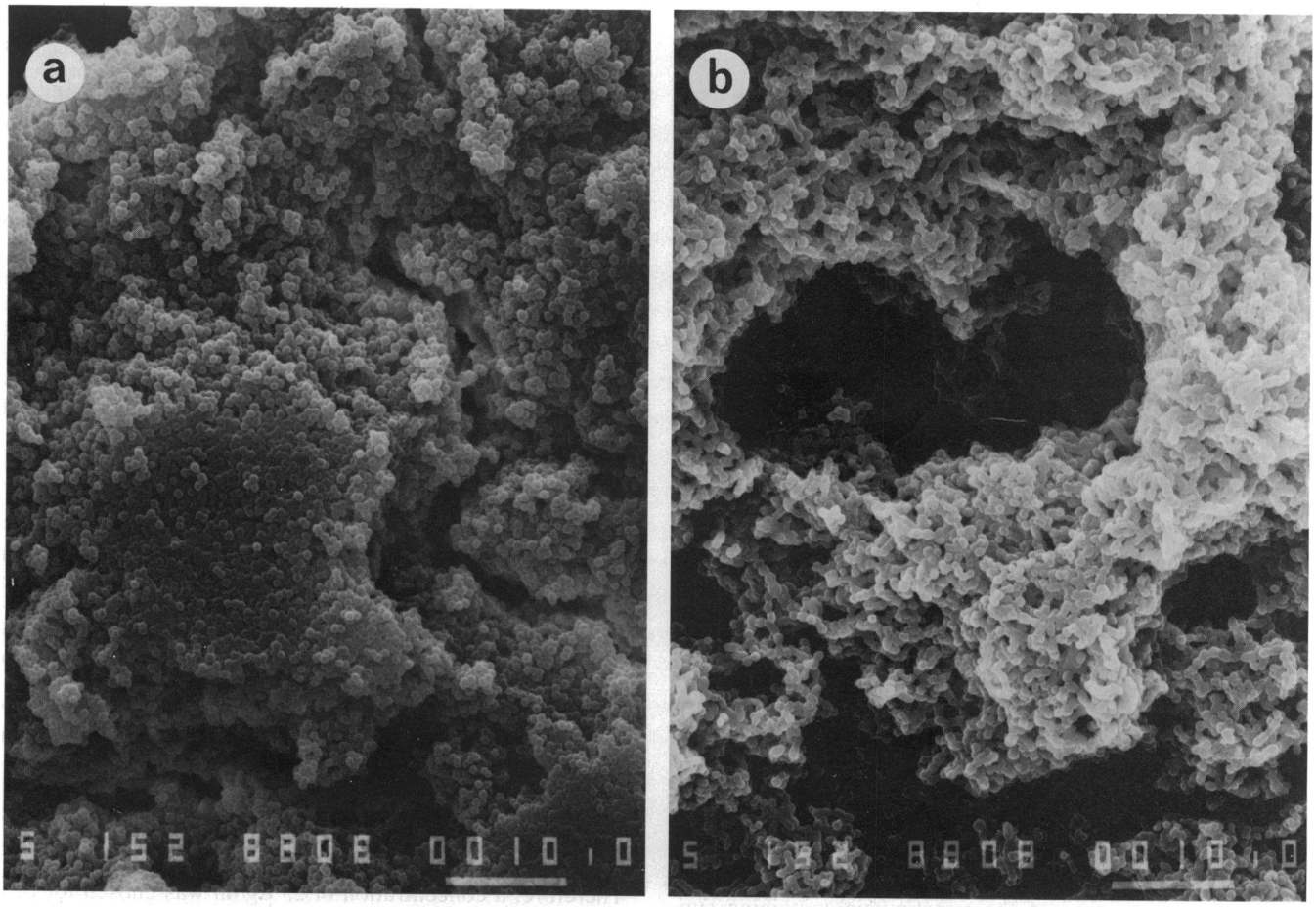

FIG. 2. (a) SEM of *S. epidermidis* biofilm before antibiotic exposure. Bar = 10 μm. (b) SEM of *S. epidermidis* biofilm after 10 continuous days of exposure to 25 μg of vancomycin hydrochloride per ml. Bar = 10 μm.

Staphylococcus aureus ATCC 29213 was used as a quality control organism; the MIC and MBC of vancomycin hydrochloride for this organism were determined to be 1.5 μg/ml by the described method in Mueller-Hinton broth and synthetic dialysis effluent medium.

Effect of inoculum size and age of silicone biofilm on vancomycin bactericidal activity. Synthetic dialysis effluent medium, with and without 25 μg of vancomycin hydrochloride per ml, was inoculated with 3.6×10^5 CFU of *S. epidermidis* per ml prepared from a 72-h synthetic dialysis effluent suspension and was incubated at 35°C. Viable cell counts were done after 24 h on vancomycin-containing and vancomycin-free suspensions.

Statistical analysis. The Student *t* test (double sided) was used to compare differences between mean bacterial population values. Analysis was done on $\log_{10}$-transformed data. *P* values of <0.05 were considered significant.

RESULTS

Biofilm development. Silicone disks were seeded initially with a mean viable cell density of 5.21 ± 1.66 (standard error) $\log_{10}$ CFU/cm^2 and a corresponding total cell density of 7.90 ± 1.24 $\log_{10}$ cells per cm^2. By 72 h of continuous flow of synthetic dialysate effluent, the viable cell count had increased to 7.37 ± 1.62 $\log_{10}$ CFU/cm^2, and the total cell count had increased to 9.55 ± 1.42 $\log_{10}$ cells per cm^2. Scanning electron micrographs of the 72-h disks revealed a fairly confluent coverage of the surface with dense multiple-layer masses of cocci surrounded by an amorphous material (Fig. 2a).

Vancomycin treatment of biofilm. Both the viable and total biofilm cell populations of silicone disks placed in control test tubes (no vancomycin) were maintained during the peritoneal model phase of the study (Fig. 3 and 4). Mean viable cell counts ranged from 7.57 ± 1.42 to 7.89 ± 1.42 $\log_{10}$ CFU/cm^2 during this 10-day period, whereas mean total biofilm cell counts ranged from 9.80 ± 1.02 to 10.41 ± 1.13 $\log_{10}$ cells per cm^2. No significant differences were observed in mean viable or total biofilm counts between days 0 and 10 ($P > 0.05$) for the control group. Mean viable cell counts of the suspension population of the antibiotic-free synthetic dialysis effluent medium in which disk-adherent biofilms had been suspended attained 7.76 ± 1.42 $\log_{10}$ CFU/ml after the initial 24 h of incubation. The suspension population was evident as turbidity. After each successive daily transfer of these control disks into sterile, antibiotic-free synthetic dialysis effluent medium, the viable suspension population continued to consistently reach levels of about 7.0 to 7.7 $\log_{10}$ CFU/ml at the end of each 24-h incubation period.

Unexpectedly, both the viable and total biofilm cell populations of vancomycin-exposed silicone disks also were maintained during the 10-day exposure period (Fig. 3 and 4). Mean viable biofilm cell counts ranged from 6.58 ± 1.51 to 7.37 ± 1.42 $\log_{10}$ CFU/cm^2, whereas mean total cell counts varied between 8.88 ± 1.13 and 9.78 ± 0.89 $\log_{10}$ cells per

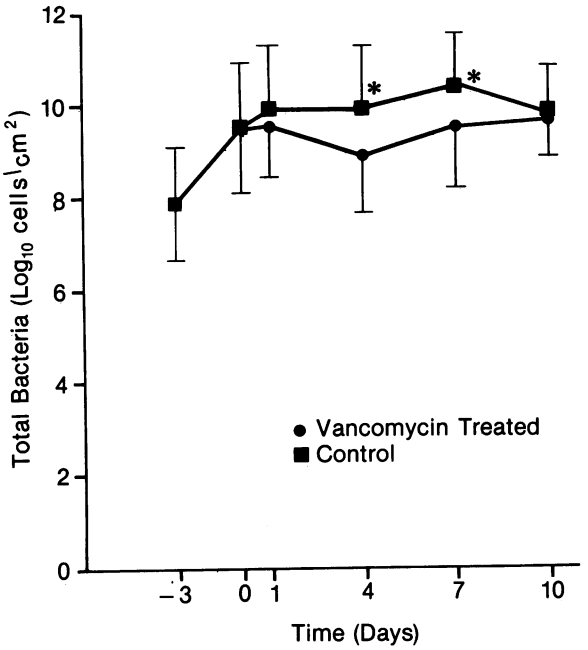

FIG. 3. Total cell population density of *S. epidermidis* biofilm grown in a flowing-medium biological sampling device for 3 days (−3 to 0), followed by 10 days in a static-medium peritoneal model. Vancomycin treatment of the test group was initiated on day 0. *, $P < 0.05$.

cm^2. Again, no significant differences were seen in mean viable or total biofilm cell counts between days 0 and 10 ($P > 0.05$). In contrast to the control disks, the mean viable suspension population of the vancomycin-containing synthetic dialysis effluent medium attained 5.34 ± 1.42 $\log_{10}$ CFU/ml after 24 h of incubation, being therefore greater than 2 logarithmic orders of magnitude below that of the antibiotic-free medium. These solutions remained nonturbid. After each successive daily transfer of silicone-adherent biofilms into fresh sterile vancomycin-containing synthetic dialysis effluent medium, a viable suspension population of about 4.7 to 5.0 $\log_{10}$ CFU/ml was present at the end of each 24-h incubation period.

Differences between vancomycin-exposed and control viable biofilm populations were significant only at days 1 and 4 after the initiation of vancomycin treatment (Fig. 4). Statistically significant differences between antibiotic-exposed and control total biofilm cell populations were observed after days 4 and 7 (Fig. 3).

SEM examination of vancomycin-treated silicone disks after 10 days of treatment (Fig. 2b) revealed a biofilm with a morphology similar to that seen before antibiotic exposure.

Susceptibility testing. The MIC and MBC of vancomycin for the *S. epidermidis* strain used were 3.125 and 6.25 μg/ml, respectively, with an inoculum of 10^5 CFU/ml from an early-stationary-phase suspension. Suspensions used to seed silicone disks at the beginning of each experiment were also in the early stationary phase.

The MICs of vancomycin for biofilm homogenates were also 3.125 μg/ml. In contrast, however, the MBCs for the homogenates were consistently >400 μg/ml and thus equivalent to >128-fold of the corresponding MICs.

Of interest is the observation that MBCs for suspension organisms obtained from the 72-h effluent of the sampling device ranged from 32- to 128-fold higher than the corresponding MIC of 3.125 μg/ml. Throughout the study, identical MICs and MBCs were recorded in synthetic dialysis effluent medium as compared with Mueller-Hinton broth.

Effect of inoculum size and age of silicone disk biofilm on vancomycin bactericidal activity. The mean viable biofilm density of 72-h disks employed for vancomycin treatment was 2.34×10^7 CFU/cm^2. The mean viable biofilm cell count per disk, therefore, can be calculated as 6.7×10^6 CFU. When an approximately equivalent inoculum of 3.6×10^6 CFU was inoculated into 10 ml of synthetic dialysis effluent, a viable population of 2.77×10^7 CFU/ml resulted after 24 h of incubation at 35°C. In contrast, the viable population of the vancomycin-containing synthetic dialysis effluent had decreased to 9.49×10^2 CFU/ml, indicating that neither inoculum size nor age significantly contributed to the apparent inability of vancomycin to kill the silicone-associated biofilm.

DISCUSSION

In this study, an *S. epidermidis* biofilm was developed on a silicone elastomer in an in vitro system modeling the microbial colonization of a peritoneal catheter. Subsequently, the biomaterial-adherent biofilm was exposed to 25 μg of vancomycin hydrochloride per ml for 10 days to evaluate the ability of this antibiotic to kill the biofilm-encased bacteria. There is still considerable controversy about the preferred route of administration of vancomycin in continuous ambulatory peritoneal dialysis-associated peritonitis (1, 4, 19, 32). However, average concentrations of dialysate vancomycin reported in the scientific literature, irrespective of the therapeutic regime selected, range from 1 to 10 μg/ml, with peaks that usually do not exceed 25 μg/ml. Therefore, a concentration of 25 μg/ml was chosen for this experiment as a therapeutically realistic but severe challenge to the biofilm, while ensuring that the concentration present

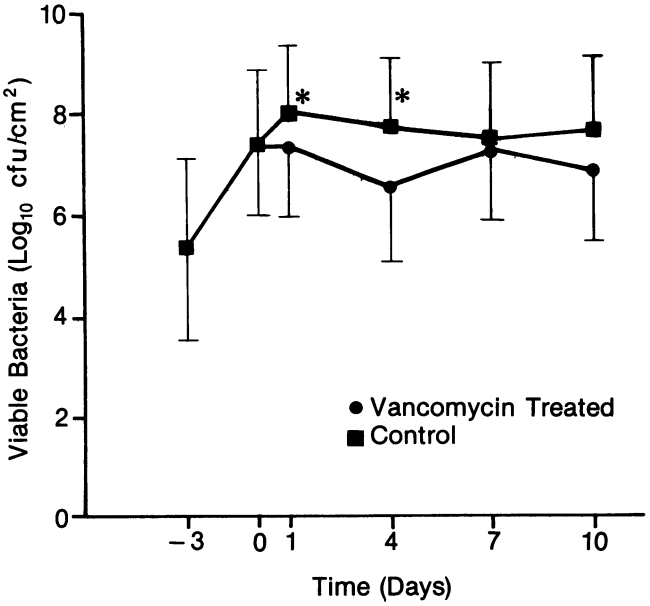

FIG. 4. Viable cell population density of *S. epidermidis* biofilm grown in a flowing-medium biological sampling device for 3 days (−3 to 0), followed by 10 days in a static-medium peritoneal model. Vancomycin treatment of the test group was initiated on day 0. *, $P < 0.05$.

was well above the MIC or MBC for the test organism, as determined by conventional methods.

Although the viable staphylococcal biofilm population decreased during the initial vancomycin treatment phase of this study relative to the control biofilm population, after 7 to 10 days of antibiotic treatment there were essentially no differences between the two populations. In addition, after 10 days of continuous vancomycin exposure, viable cell counts of the biofilm population were less than 0.5 $\log_{10}$ lower than those at the start of antibiotic treatment, and total cell counts had not significantly changed at all. During the course of the experiment, total cell counts paralleled viable cell counts, and at 10 days there were no differences in the total cell counts between vancomycin-exposed and control biofilms. These data indicate that the biofilm-encased *S. epidermidis* demonstrated virtually no response to vancomycin treatment at levels approximately fourfold above the MBC, as determined by conventional macrobroth dilution methods, even if the exposure time was extended for 10 days.

It is of interest that the control biofilm did not significantly increase in either total or viable cell counts after 24 h in the static peritoneal model, indicating that cell division was not rapid, if occurring at all. In contrast, the biofilm viable cell count did steadily increase during the biofilm development portion of the experiment in which chemostat parameters were used. Cell division within the biofilm in the static peritoneal model may have been limited because of diffusion limitations on the transport of nutrients and oxygen to the biofilm surface as a result of a relatively thick mass transfer boundary layer (5). In addition to nutrient limitation effects on biofilm proliferation, the same phenomenon could also apply to vancomycin at the biofilm-liquid interface, resulting in decreased antibiotic availability from the bulk surrounding solution (11).

The relationship between the metabolic activity and specific growth rate of biofilm-encased *S. epidermidis* cells and their apparent tolerance to vancomycin treatment observed in this study is unclear. There is controversy about the metabolic state of bacteria within a biofilm (13, 16, 20), and its relevance to antibiotic tolerance was beyond the scope of this study.

MICs and MBCs for the strain of *S. epidermidis* used, when grown in liquid suspension, were 3.125 and 6.25 μg/ml, respectively, indicating a susceptible strain and giving no indication of tolerance (33). In contrast, extreme tolerance was observed with an inoculum of homogenized biofilm. This effect was also observed for suspension bacteria in the effluent of the biological sampling device at the 72-h sampling period. Artifacts caused by inoculum size could not be implicated, as approximately 10^5 CFU/ml were consistently used for MIC testing. Many of these suspension bacteria probably originated from the device-associated biofilm, thereby resembling the resident biofilm population in terms of growth rate, metabolic state, and morphology.

The ability of such bacterial populations to exhibit tolerance may be explained by the observations of Cozens et al. (7), who demonstrated that many bactericidal β-lactam antibiotics are ineffective against slowly growing organisms. Alternatively, the phenotypic morphology of these biomaterial-adherent organisms, evident as dense aggregates of bacteria and extracellular material, may hinder the diffusion of an effective antibiotic concentration to the antimicrobial site of action (10, 17, 35).

The observation that a staphylococcal biofilm persisted for 10 days when continuously exposed to vancomycin at a 25-μg/ml concentration may be of significant relevance to the treatment of continuous ambulatory peritoneal dialysis-associated peritonitis. The latter antibiotic concentration is higher than that normally maintained in the peritoneum when administered for the treatment of gram-positive bacterial peritonitis and failed to successfully kill significant numbers of biofilm-encased *S. epidermidis*. Traditional antibiotic regimens of vancomycin therapy may not always be sufficient to eradicate catheter-associated biofilms, potentially leading to relapsing peritonitis with the same species upon cessation of antibiotic therapy. The model described in this paper can be used to further investigate the ability of alternative forms of antibiotic therapy to eradicate biomaterial-associated biofilms.

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