

## Impact of Bacterial Biofilm Formation on In Vitro and In Vivo Activities of Antibiotics

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**The impact of bacterial adherence on antibiotic activity was analyzed with two isogenic strains of *Staphylococcus epidermidis* that differ in the features of their in vitro biofilm formation. The eradication of bacteria adhering to glass beads by amikacin, levofloxacin, rifampin, or teicoplanin was studied in an animal model and in a pharmacokinetically matched in vitro model. The features of *S. epidermidis* RP62A that allowed it to grow on surfaces in multiple layers promoted phenotypic resistance to antibiotic treatment, whereas strain M7 failed to accumulate, despite initial adherence on surfaces and growth in suspension similar to those for RP62A. Biofilms of *S. epidermidis* M7 were better eradicated than those of strain RP62A in vitro (46 versus 31%;  $P < 0.05$ ) as well as in the animal model (39 versus 9%;  $P < 0.01$ ).**

Antibiotic therapy against an established biofilm often fails without removal of the infected implant, despite the use of drugs which are highly active in standard in vitro susceptibility tests (8). In contrast to antibiotic therapy of many bacterial infections, standard in vitro susceptibility tests are not predictive of the therapeutic outcome of device-related infections (26).

Adhesion to biomaterials is assumed to be a crucial step in the pathogenesis of foreign body-related infections. *Staphylococcus epidermidis* represents the most prominent organism responsible for device-related infections (12, 19), and most *S. epidermidis* strains are able to attach to polymer surfaces, albeit with quantitative differences between strains (6, 10, 17, 24). Two phases can be kinetically differentiated during the establishment of a biofilm, as documented by scanning electron microscopy. A rapid initial attachment of *S. epidermidis* cells to the polymer surface is later followed by the accumulation of cells in multilayered cell clusters and glycocalyx production (18). The findings of different investigators suggest that extracellular slime does not play an important role in the early phase of attachment of coagulase-negative staphylococci to a biomaterial surface (7, 10, 17, 23). Colonization of foreign bodies by coagulase-negative staphylococci is likely to be a dynamic process, and the factors that mediate initial bacterial adherence may not be the same as those favoring bacterial persistence, multiplication, and ultimately, clinical infection.

The present study focuses on the impact of bacterial adherence on the in vivo and in vitro activities of antibiotics by considering a pair of isogenic strains that differ with respect to the features of their accumulation on surfaces. In particular, it was the aim of the study (i) to observe the impact of differences in bacterial biofilm formation on the phenotypic resistance of *S. epidermidis* to the bactericidal activity of antibiotics and (ii) to compare the results obtained with an animal model (30) and those obtained with an in vitro model (25) of treatment of bacterial biofilms with antimicrobial agents.

### MATERIALS AND METHODS

**Bacteria.** Two strains of *S. epidermidis* have been used: a well-described adherence-positive wild-type strain (*S. epidermidis* RP62A [ATCC 35984] [4, 14, 15]) and its adherence-negative mutant (*S. epidermidis* M7, described by Schumacher Perdreau et al. [21]).

**Drug susceptibility.** MICs and minimum bactericidal concentrations (MBCs) were determined with both suspended and adherent bacteria. MICs were determined in both (i) tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) supplemented with 50  $\mu\text{g}$  of  $\text{Ca}^{2+}$  per ml and 25  $\mu\text{g}$  of  $\text{Mg}^{2+}$  per ml (TSB-S) and (ii) phosphate-buffered saline (PBS)-GCP (PBS-GCP is 2.38 g of  $\text{Na}_2\text{HPO}_4$ , 0.19 g of  $\text{KH}_2\text{PO}_4$ , 8.0 g of NaCl, 0.9 g of glucose, 1.0 g of Casamino Acids, and distilled water to 980.0 ml, which was separately autoclaved; 20.0 ml of sterile human plasma was then added to the mixture). The suspended bacteria were tested by a macrodilution method with a standard inoculum of  $10^6$  CFU/ml (1). MBCs were determined as described by Amsterdam (2). The lowest antibiotic concentration that reduced the inoculum by  $\geq 99.9\%$  was defined as the MBC. The susceptibility of adherent bacteria was tested by a previously described macrodilution method (25, 29) with an inoculum of  $5.5 \times 10^5$  CFU/bead. The MBCs were the lowest antibiotic concentrations that killed  $\geq 99.9\%$  of the adherent bacteria.

**Animal model.** The previously described guinea pig tissue cage model was used (30). In brief, four sterile polytetrafluoroethylene (Teflon) tubes (32 by 10 mm) perforated with 130 regularly spaced 1-mm-diameter holes (Ciba-Geigy Ltd.) were each filled with six sinter-glass beads (Sikug 023/300 A; Schott Schleifer AG, Muttenz, Switzerland) and were aseptically implanted in the flanks of albino guinea pigs (weight, 600 to 1,100 g). Experiments started after complete healing of the wound (3 weeks after surgery). Prior to each experiment, the interstitial fluid that accumulated in the tissue cages was checked for sterility. Tissue cages were infected by local inoculation of about  $10^7$  CFU. Antibiotic therapy was started 16 h after inoculation. At this time the number of suspended bacteria averaged  $5.99 \pm 0.3 \log_{10}$  CFU/ml for strain M7 and  $6.02 \pm 0.44$  CFU/ml for strain RP62A. The kinetics of each drug were measured in 10 to 12 tissue cage fluid samples from three animals. The kinetics were determined at up to seven time points (time zero to 24 h). In this report only the peak and the trough levels are given. The average time course of the measured concentrations was used as a reference to stimulate the kinetics in vitro. Therefore, pharmacokinetic measurements were performed for a total of 12 animals.

**In vitro model.** A previously described one-compartment pharmacokinetic model was used in the present study (22, 25). This model allows periodic assessment of the bactericidal effect of antibiotic dosing against both adherent and suspended bacteria. A peristaltic pump continuously transported sterile culture medium from the reservoir into the 17-ml culture compartment resulting in an exponential decrease in the drug concentration. The bacteria were exposed in the model to oscillating drug levels simulating the kinetics determined in the cages implanted in the animals.

The system was filled with a special culture medium referred to here as PBS-GCP. In contrast to standard culture medium this medium supports growth at only a limited rate and results in a reduced final bacterial density. The generation time in this medium was 64 min for strain M7 and 81 min for strain RP62A, whereas a generation time of 26 min was observed for both strains in TSB.

The intention was to achieve in the in vitro model an inoculum of suspended bacteria with numbers similar to the numbers in vivo at the onset of treatment.

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TABLE 1. Dosing and kinetics in the in vivo and in vitro models

Antibiotic	Dose (mg/kg of body weight) in vivo and route of administration	Peak level in serum in vivo ( $\mu\text{g/ml}$ )	Level in cages ( $\mu\text{g/ml}$ )		Level in in vitro culture compartments ( $\mu\text{g/ml}$ )		$t_{1/2}$ in vitro (h) <sup>d</sup>
			Peak <sup>a</sup>	Trough <sup>b</sup>	Peak <sup>c</sup>	Trough <sup>b</sup>	
Amikacin	20, i.m. <sup>e</sup>	46 ( $n = 3$ )	7.42 ( $n = 10$ )	2.72 ( $n = 10$ )	7.4	2.8	7.93
Levofloxacin	10, i.p.	5.22 ( $n = 3$ )	1.47 ( $n = 12$ )	0.27 ( $n = 12$ )	1.5	0.28	4.18
Rifampin	25, i.p. <sup>f</sup>	14.5 ( $n = 3$ )	6.32 ( $n = 12$ )	0.63 ( $n = 12$ )	6.3	0.66	2.45
Teicoplanin	6.6, i.p.	15 ( $n = 3$ )	14.1 ( $n = 12$ )	3.7 ( $n = 12$ )	14.1	3.7	4.18

<sup>a</sup> Peak levels in the infected tissue cages were achieved with rifampin and teicoplanin after 4 h, with amikacin after 1 h, and with levofloxacin after 2 h.

<sup>b</sup> Trough levels in the infected tissue cages and culture compartments were achieved with all antibiotics after 12 h.

<sup>c</sup> Peak levels in the in vitro culture compartments were achieved with rifampin and teicoplanin after 4 h of continuous infusion, with amikacin after 1 h of continuous infusion, and with levofloxacin after 2 h of continuous infusion (concentrations were estimated by measuring the dilution profile of a test substance).

<sup>d</sup>  $t_{1/2}$ , half-life.

<sup>e</sup> i.m., intramuscularly.

<sup>f</sup> i.p., intraperitoneally.

To obtain such an in vitro inoculum, glass beads were exposed to the bacteria in a flask for 16 h before the onset of treatment. This simulated the 16-h delay between in vivo inoculation and the start of therapy. Sterile sinter-glass beads (Sikug 023/300 A; Schott Schleifer AG) were placed for 16 h in a bacterial suspension of 25 ml of PBS-GCP inoculated with three bacterial colonies taken from a blood agar plate (working cultures). The beads covered with bacterial biofilm were separated from the PBS-GCP of the overnight culture by filtration and were rinsed with 10 ml of sterile saline solution to wash off the bacteria suspended within the broth carried over from the culture. Subsequently, 10 beads were placed into the culture compartment with a sterile surgical forceps 30 min before the start of antibiotic treatment. The inocula in the suspensions in the model present at the beginning of antibiotic dosing averaged  $5.61 \pm 0.37$  and  $5.20 \pm 0.26 \log_{10}$  CFU/ml for strains M7 and RP62A, respectively. The number of adherent bacteria per glass bead averaged  $5.91 \pm 0.48$  and  $5.65 \pm 0.25 \log_{10}$  CFU/bead for strains M7 and RP62A, respectively. Control experiments demonstrated stationary-phase conditions. The number of suspended bacteria as well as the number of adherent bacteria of both strains did not change by more than 0.66 log over an incubation period of 48 h in the in vitro model. Each drug and both strains have been tested with three animals with four tissue cages containing a total of 24 beads. The numbers of CFU in one animal were determined at the beginning of the treatment, and the numbers of CFU in a second animal not treated with an antibiotic were determined at the same time point. Since only the data for animals with complete eradication of all microorganisms are reported (see Table 2), the data for the control animals are not presented. Overall, drug efficacy was evaluated with 24 animals (3 animals per drug and strain). Since each bead was processed separately 72 individual microbiological determinations were performed per drug and strain. Due to the need to restrict the use of animals, we believe that it would not have been justified to use more animals.

**Antibiotic concentrations and dosage regimens.** Table 1 summarizes the dosing of the antibiotics in vivo and the concentrations achieved in serum and tissue cage fluid. The kinetics of each drug have been measured in 10 to 12 tissue cage fluid samples from three animals. The kinetics were determined at up to seven time points (time zero to 24 h). In this report only the peak and trough levels are given. The average time course of the measured concentrations was used as a reference to simulate the kinetics in vitro. Therefore, pharmacokinetic measurements were obtained for a total of 12 animals. The peak and trough concentrations obtained in vitro are also listed. The concentrations determined in the samples from the animals in the in vivo study were determined in antibiotic medium 1 (Difco Laboratories) by agar diffusion bioassays. *Bacillus subtilis* 0453-52-9 (Difco) was used as the test strain. Standard curves were generated from pooled heat-inactivated guinea pig serum with a final concentration of 50%. Results were calculated by microcomputer-assisted exponential regression analysis (Hewlett-Packard HP41V). The concentrations achieved in vitro were defined according to the concentrations obtained in vivo in the infected tissue cages. A one-compartment kinetic model was simulated in vitro on the basis of the in vivo observations over the two 12-h dosing intervals (Table 1). Concentration-time profiles for all four drugs were calculated, and the presence of the calculated concentration profiles within the culture compartments was confirmed by spectrophotometric concentration measurements by using fluorescein sodium as the test substance (PBS [pH 7.4], 23°C, 490 nm;  $r^2 = 0.9996$ ; Stasar III; Gilford Instrument Laboratories Inc., Oberlin, Ohio). The kinetics of the in vitro system were determined every 0.5 h over the entire dosing interval. The mean intrasay and interassay coefficients of variation were less than 5%; the measured concentrations of all four drugs averaged  $101\% \pm 5\%$  of the respective target values.

**Quantification of bactericidal activity.** (i) **In vivo.** Six beads were removed from each cage at each sampling point. The number of bacteria adhering to the beads was determined as described previously (29). Each bead was placed on a filter with a sterile forceps and was washed twice with saline. The number of

bacteria adhering to the beads was determined by placing the washed glass beads in 2 ml of physiological saline containing EDTA (0.15%) and Triton X-100 (0.1%), followed by vigorous vortexing three times for 15 s each time to remove the adhering bacteria. Thereafter, the tubes were placed in an ultrasonic bath and sonicated for 3 min at 120 W (Labsonic 2000; Bender & Hobein, Zurich, Switzerland). The specimens containing resuspended bacteria were subsequently diluted 100- to 10,000-fold and were subcultured at 37°C onto tryptic soy agar plates. The detection limit was 20 CFU per bead. The term "sterile" is defined here as no bacterial growth in subcultures of individual beads.

(ii) **In vitro.** Each bead was removed from the compartment after 24 h of antibiotic treatment. The bacteria adhering to the sinter-glass bead were detached by vortexing as described previously (25). The detection limit was 40 CFU per bead. The term "sterile" is defined here as no bacterial growth in subcultures of individual beads.

**Data analysis.** Probabilities ( $P$  values) of  $<0.05$  were considered statistically significant. The chi-square test was used for analysis of the frequency of bacterial eradication.

## RESULTS

**Antibiotic efficacy against adherent bacteria.** In vivo *S. epidermidis* M7 was eradicated from 37 of 96 beads (39%), whereas *S. epidermidis* RP62A was eradicated from 9 of 96 beads (9%) ( $P < 0.01$ ) (Table 2). The difference between the eradication of the two strains was less pronounced in vitro but was still statistically significant (46 of 100 versus 31 of 100 beads;  $P < 0.05$ ). In vivo, rifampin eradicated both strains of glass bead-adherent bacteria more frequently than each of the other three drugs ( $P < 0.05$ ), (Table 2). Compared to the in vitro activities of the other three drugs, rifampin was most efficient against strain RP62A ( $P < 0.05$ ) (Table 2). Compared to rifampin, levofloxacin was insignificantly less active in vitro against strain M7 but was considerably less active against strain RP62A ( $P < 0.05$ ).

**Impact of culture medium and bacterial adherence on MBCs.** The susceptibilities of both strains were determined by

TABLE 2. Eradication of adherent bacteria of two isogenic strains of *S. epidermidis* in an in vitro and in an in vivo model

Drug	No. of sterile beads/total no. of beads studied			
	In vitro model		In vivo model	
	RP62A	M7	RP62A	M7
Amikacin	0/25	0/25	0/24	6/24 <sup>a</sup>
Levofloxacin	9/25	21/25 <sup>a</sup>	1/24	8/24 <sup>a</sup>
Rifampin	22/25	25/25	8/24	18/24 <sup>a</sup>
Teicoplanin	0/25	0/25	0/24	5/24 <sup>a</sup>

<sup>a</sup> The statistical significance of the eradication rate between the two strains was determined, and the difference was significant ( $P < 0.05$ ).

TABLE 3. Antibiotic activity against an isogenic pair of *S. epidermidis* strains<sup>a</sup>

Strain	Drug	MIC ( $\mu\text{g/ml}$ ) for bacteria suspended in TSB-S	MBC ( $\mu\text{g/ml}$ )			
			TSB-S		PBS-GCP	
			Suspended bacteria	Adherent bacteria	Suspended bacteria	Adherent bacteria
<i>S. epidermidis</i> RP62A	Amikacin	16 <sup>a</sup>	64	>256	>256	>256
	Levofloxacin	0.125	0.5	0.5	0.5	1
	Rifampin	<0.008	<0.008	>2	0.016	2
	Teicoplanin	2	4	64	4	>128
<i>S. epidermidis</i> M7	Amikacin	16	64	>256	>256	256
	Levofloxacin	0.125	0.25	1	0.5	1
	Rifampin	<0.008	0.008	>2	<0.008	2
	Teicoplanin	4	4	64	4	>128

<sup>a</sup> The MICs and MBCs for suspended and adherent bacteria were measured in supplemented TSB (TSB-S) and a special slow growth medium (PBS-GCP).

the MIC macrodilution method as well as by four different MBC methods (Table 3). Only limited differences were observed between the MBCs determined in TSB and those determined in PBS-GCP for either suspended or adherent pathogens. However, major differences were noted in a comparison of the MBCs for adherent and suspended bacteria, particularly with rifampin and teicoplanin. The poor activity of amikacin in both the in vivo and the in vitro models reflected the fact that peak levels in the culture compartments were consistently lower than the MICs and MBCs for both strains (Tables 1 and 3). In contrast, the concentrations of the other three compounds in the culture compartments were above the MICs and MBCs determined for suspended bacteria for almost the entire dosing interval. The peak levels but not the trough levels of levofloxacin were above the MBCs determined for adherent bacteria. Similarly, the peak levels but not the trough levels of rifampin were above the MBC for the adherent bacteria determined in PBS-GCP. For teicoplanin even the peak levels were below the MBCs for the adherent bacteria determined in PBS-GCP and in TSB-S.

## DISCUSSION

This study shows an impaired antimicrobial eradication of a biofilm-forming strain of *S. epidermidis* compared to the antimicrobial eradication of its biofilm-negative mutant. This phenotypic resistance relates to a multilayered growth on surfaces and was observed despite similar initial levels of adherence on surfaces and similar levels of growth in suspension. The significance of adherent growth of coagulase-negative staphylococci has previously been assessed both in vitro and in an endocarditis model (20, 27). The in vitro study considered various culture conditions that closely mimic the clinical situation with both important clinical isolates and isolates from the skin of volunteers (27). The in vivo study considered the pair of isogenic strains used in the present study (20). In both investigations, the isolates that exhibited accumulative growth when cultured in TSB did not differ with respect to biofilm formation when cultured in the presence of serum. In contrast, the data obtained in the present study suggest that despite the addition of serum to the culture medium, differences in the bactericidal activities of antimicrobial agents can be detected in vivo as well as in vitro.

The enhanced activities of levofloxacin and rifampin compared with the activities of the two other drugs in both the in vivo and the in vitro models are consistent with increased ratios of peak concentrations versus MBCs for adherent bacteria.

The MBCs were comparable to the average concentrations obtained in the cages and culture compartments. The MICs and MBCs determined for suspended bacteria were less predictive for the assessment of efficacy in both models. These findings are consistent with those of previous studies documenting that successful treatment of experimental device-related infections cannot be predicted when the trough antibiotic levels exceed the MIC at the site of infection (29). In contrast, in vivo efficacy was predicted if the MBC for the organism in the stationary phase was in the susceptible range and if glass-adherent staphylococci were killed by low drug concentrations.

An interesting difference was noted when comparing the MBCs for suspended bacteria versus those for adherent bacteria for both strains. In contrast to amikacin, rifampin, and teicoplanin, the in vitro activity of levofloxacin was compromised only to a very limited extent by bacterial adherence. The reasons for this observation are not clear.

A recent investigation that considered the same isogenic strains of *S. epidermidis* used in this study provided biochemical and functional evidence that a 140-kDa protein present in extracellular material plays a role in the accumulative growth of *S. epidermidis* RP62A (11). This protein is lacking in mutant M7. The protein could be isolated only by using sessile growth conditions, possibly reflecting an important property of staphylococci, that is, that they can rapidly change specific phenotypic features (phase variants) (4). Various factors that contribute to the primary attachment of *S. epidermidis* cells to surfaces have been described and isolated. Among these factors, the capsular polysaccharide adhesin (16, 24), the slime-associated antigen (3, 5), and the polysaccharide intercellular adhesin (9, 13, 28) are of particular interest. One may ask of what importance these factors are, considering the impact of culture conditions and recognizing the fact that bacterial growth and persistence must occur in vitro as well as in vivo in changing, hostile environments. So far, the significance of these factors studied either in vitro or ex vivo has not yet been sufficiently documented in a clinical context. Further studies are needed to evaluate a possible correlation between the presence of these factors and the severity of device-associated infections. Only a limited number of investigations have assessed the isolated effects of specific factors in animal models, and even fewer data on the significance of these factors for antibiotic therapy are available.

In conclusion, the findings presented here suggest that differences in the accumulative growth on surfaces may present themselves as phenotypic resistance to antibiotic treatment of biofilm-forming *S. epidermidis*.

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