MAJOR ARTICLE

Use of the Quorum-Sensing Inhibitor RNAIII-Inhibiting Peptide to Prevent Biofilm Formation In Vivo by Drug-Resistant *Staphylococcus epidermidis*

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***Staphylococcus epidermidis* is a frequent cause of infections associated with foreign bodies and indwelling medical devices. The bacteria are capable of surviving antibiotic treatment through encapsulation into biofilms. RNAIII-inhibiting peptide (RIP) is a heptapeptide that inhibits *S. aureus* pathogenesis by disrupting quorum-sensing mechanisms. In this study, RIP inhibited drug-resistant *S. epidermidis* biofilm formation through a mechanism similar to that evidenced for *S. aureus*. RIP is synergistic with antibiotics in eliminating 100% of graft-associated in vivo *S. epidermidis* infections, which suggests that RIP may be used to coat medical devices to prevent staphylococcal infections. Disruption of cell-cell communication can prevent infections associated with antibiotic-resistant strains.**

Staphylococcus epidermidis is a gram-positive, coagulase-negative staphylococcal bacterium. It is a common inhabitant of skin and is the most prevalent of the staphylococcal species. Once considered to be harmless, it is now known to be a frequent cause of infections related to foreign bodies and indwelling medical devices [1]. In the United States, 1 million nosocomial infections each year are related to infections caused by biofilm formed on implanted devices [2]. Infections of central venous catheters, urinary catheters, prosthetic heart valves, orthopedic devices, and even contact lenses are associated with bacterial biofilm formed on the material's surface [3, 4]. The outcome of these infections can be longer hospitalization, surgery, and death [5, 6]. Moreover, tissues in proximity to the implanted device could sustain collateral damage from immune complexes and neutrophils, leading to an extensive necrosis and inflammation [3].

Finding an effective antibiotic treatment for *S. epidermidis* infection has been difficult, because this organism has the capacity to form a biofilm, which is often resistant to antimicrobial agents [3, 7–9]. The antibiotic concentration required to kill bacteria in the biofilm is 100–1000 times higher than that needed to kill the same species in suspension [9]. Because of an increase in methicillin-resistant strains, recent prophylactic strategies have included precoating of devices with glycopeptide antibiotics [10, 11], but the recent appear-

Received 3 June 2002; revised 1 October 2002; electronically published 7 February 2003.

Presented in part: Center for Biofilm Engineering Technical Advisory Conference, Bozeman, Montana, 22–25 July 2002.

Financial support: American Heart Association, Western States Affiliate (to N.B.).

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The Journal of Infectious Diseases 2003; 187:625–30

0022-1899/2003/18704-0012$15.00

ance of *S. epidermidis* strains resistant to vancomycin [12, 13] and teicoplanin [13, 14] has created the need for alternative strategies.

Studies have focused on developing new prosthetic materials that reduce adhesion or survival of bacteria [15, 16] and on better understanding of the mechanisms of bacterial adhesion and biofilm formation with the goal of identifying new therapeutic targets. Several hypotheses have been formulated on the basis of experimental observations [3, 7, 8, 17, 18]. One of the most intriguing is that resistance of biofilm to antimicrobial agents is acquired as a multicellular strategy that relies on exchange of chemical signals between cells in a process known as "quorum sensing" [19]. Interfering with this mechanism of bacterial cell-cell communication can provide a novel approach to prevent biofilm formation. Recently, a peptide was described that inhibits biofilm formation and diseases caused by *S. aureus* [20–24]. The heptapeptide, which was originally isolated from postexponential supernatants of *S. xylosus* [23], is now made in its amide form as a synthetic 7-aa molecule (YSPWTNF-NH2) termed "RNAIII-inhibiting peptide" (RIP).

RIP inhibits cell adhesion and biofilm formation [23, 24] and inhibits the activity of the gene locus *agr,* thus preventing the production of a regulatory RNA molecule, RNAIII, that regulates the production of toxins and influences the pathogenesis of *S. aureus* [23, 25]. The mechanism through which RIP inhibits quorum-sensing mechanisms involves inhibition of the phosphorylation of a protein called "target of RNAIII activating protein" (TRAP) [22]. We found evidence of the presence of TRAP in *S. epidermidis,* leading us to form the hypothesis that similar quorum-sensing mechanisms are present in *S. aureus* and *S. epidermidis* and that RIP may interfere with biofilm formation and infections caused by both species. Here we explore that hypothesis and report our results.

MATERIALS AND METHODS

Bacteria. Methicillin-susceptible *S. epidermidis* (MSSE; ATCC 12228) was purchased from Oxoid. Clinical isolates of methicillin-resistant *S. epidermidis* (MRSE) and *S. epidermidis* with intermediate resistance to glycopeptides (GISE) were obtained from the Institute of Infectious Diseases and Public Health, University of Ancona, Ancona, Italy. A clinical isolate of the *S. epidermidis* strain Sofi was obtained from Tel Aviv University, Tel Aviv, Israel. As a control, we used *S. aureus* strain RN6390B (ATCC 55620). Bacteria were grown at 37°C in CY broth plus β-glycerophosphate (1% yeast extract, 100 m*M* NaCl, 100 m*M* sodium glycophosphate, and 0.5% glucose [pH 7.4]).

Human cells. HaCat human skin keratinocytes were grown at 37°C in a 5% CO_2 humidified incubator in bicarbonate-buffered Dulbecco's MEM supplemented with 5% fetal calf serum.

Animals. For our experiments, we used adult male Wistar rats weighing 300–350 g (Istituto Nazionale Riposo e Cura Anziani—Istituto di Ricovero e Cura a Carattere Scientifico animal facility, Ancona). Each experimental group included 15 animals.

Antibiotics. The antibiotics used were cefazolin and rifampin (Sigma-Aldrich), imipenem (Merck Sharp & Dohme), levofloxacin and teicoplanin (Hoechst Marion Roussel), mupirocin (SmithKline Beecham Pharmaceuticals), and quinupristin-dalfopristin (Aventis Pharma). Laboratory powders were diluted in accordance with manufacturers' recommendations. Solutions of drugs were freshly made on the day of assay or stored at −70°C in the dark for short periods.

RIP. The amide form of RIP (YSPWTNF-NH2) and fluorescein isothiocyanate (FITC; Sigma)–labeled RIP (Cys[S,fluorescein]-YSPWTNF-NH2) were synthesized by Neosystem (Strasbourg, France). Peptides were purified to 99% by high-performance liquid chromatography [23].

Binding of RIP to Dacron. To determine how much RIP impregnates Dacron, FITC-RIP (10 mg/L) was applied to a 1-cm^2 sterile collagen-sealed Dacron graft (Albograft; Sorin Biomedica Cardio) for 20 min at room temperature. Fluorescence in unbound solution was determined at 485 and 530 nm in a microplate fluorescence reader (FL 600) with KC4 software (Bio-Tek).

TRAP phosphorylation. To test for TRAP phosphorylation in *S. epidermidis,* in vivo phosphorylation assays were carried out as described elsewhere for *S. aureus* [22]. In brief, *S. epidermidis* cells were grown in CY broth to the early exponential phase of growth (OD_{600}, 0.2). Cells were collected by brief centrifugation and resuspended in phosphate-free buffer supplemented with radiolabeled orthophosphate with or without RIP (10 μg/10^7 cells). After a 1-h incubation period, the cells were collected by centrifugation and treated with lysostaphin, and sample buffer was added. Total cell homogenate was applied, without boiling, to a 15% SDS-PAGE gel, and the gel was autoradiographed. The same experiment was carried out using RN6390B *S. aureus* cells as a positive control.

Adherence to human cells. Bacterial cells were labeled with FITC as described elsewhere [23]. To test *S. aureus* adherence to human cells, 10^4 HaCat cells were applied to Costar 96-well cell culture polystyrene plates (Corning) for 1 day to reach confluency, and FITC-labeled bacteria (10^6 cells/well in 90 μL of PBS) were added (with and without 5 μg of RIP). Microtiter plates containing bacteria and HaCat cells were incubated for 30 min at 37°C and washed with PBS, and fluorescence was determined (at 485/530 nm) in a microplate fluorescence reader (FL 600) with KC4 software [24].

Adherence to plastic. Bacteria were grown in broth to the early exponential phase. In total, we applied 200 μL (10^8 cells) in triplicate to 96-well cell culture polystyrene plates (Corning) with or without RIP (10 μg RIP/10^7 bacteria). Cells were grown without shaking at 37°C for 3 h to allow biofilm formation.

Nonadherent cells were removed by 3 gentle washes with PBS, and adherent cells were stained by the addition of 100 μL of 0.1% safranin for 30 s. Unbound stain was removed by 3 gentle washes with PBS. Stained cells were dried for 30 min at room temperature, 100 μL of 0.1% SDS in water was added, and, after 1 h of incubation at room temperature, absorbance was read at 490 nm [24].

Induction of graft-associated infection. After rats (300–350 g) were anesthetized, the hair on their backs was shaved, and the skin was cleansed with a 10% povidone-iodine solution. We made a subcutaneous pocket on the side of the median line, using a 1.5-cm incision, and aseptically implanted a 1-cm^2 sterile collagen-sealed Dacron graft (Albograft; Sorin Biomedica Cardio) into the pocket. Immediately before implantation, the Dacron grafts were soaked for 20 min in sterile solutions of 10 mg of RIP/L of saline, in saline only, or in inactive RIP analogue (YKPETNF) as controls, with or without antibiotics (mupirocin, 100 mg/L; quinupristin-dalfopristin, 50 mg/L; levofloxacin, 30 mg/L; or rifampin, 5 mg/L). Pockets were closed by skin clips, and 1 mL of sterile saline solution with or without 2×10^7 bacteria was inoculated onto the graft surface with a tuberculin syringe to create a subcutaneous fluid-filled pocket. Some animals that had grafts soaked only with RIP or saline were injected intraperitoneally with antibiotics (cefazolin, 30 mg/kg; imipenem, 30 mg/kg; teicoplanin, 10 mg/kg; or levofloxacin, 10 mg/kg). Grafts were explanted 7 days after implantation.

Assessment of graft infection. The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 mL of PBS, and sonicated for 5 min to remove adherent bacteria from the grafts. Viable bacteria were quantitated by culturing serial 10-fold dilutions (0.1 mL) of the bacterial suspension on blood agar plates. All plates were incubated at 37°C for 48 h and evaluated for the presence of the strain. We then counted the number of colony-forming units per plate. To determine whether bacteria were efficiently removed from grafts, washed and sonicated grafts were observed under an optical microscope (Nikon Eclipse E 600; Y-THS).

Statistical analysis. Quantitative culture results for the in vivo experiments are presented as mean ± SD. Results were compared by analysis of variance of log-transformed data. In vitro adhesion assays were analyzed by Student's *t* test (Excel; Microsoft). $P \leq .05$ was considered to be significant.

RESULTS

Inhibition by RIP of* S. epidermidis *adhesion to human cells and formation of biofilm on plastic. The virulence of *S. epidermidis* often is associated with its ability to adhere to host cells and to form biofilm on medical devices. To test whether RIP can prevent *S. epidermidis* from colonizing host cells and may therefore be a candidate for therapy and prevention, FITC-labeled *S. epidermidis* were incubated, in the presence or absence of RIP, with a confluent layer of keratinocytes (HaCat cells) for 30 min. As shown in figure 1*A*, RIP significantly ($P < .05$) reduced *S. epidermidis* adherence to HaCat cells.

To test whether RIP reduces adherence and biofilm formation of *S. epidermidis* to plastic, early exponential *S. epidermidis* were grown for 3 h in polystyrene microtiter plates, and adherent cells were stained with safranin. These experimental conditions allow the formation of biofilm (as observed by atomic force microscopy; data not shown). As shown in figure 1*B*, RIP significantly reduced the number of cells that adhered to plastic. These results clearly demonstrate that RIP inhibits the adhesion of *S. epidermidis* to host cells and to plastic in vitro.

Prevention of graft-associated infection by RIP. Peritoneal

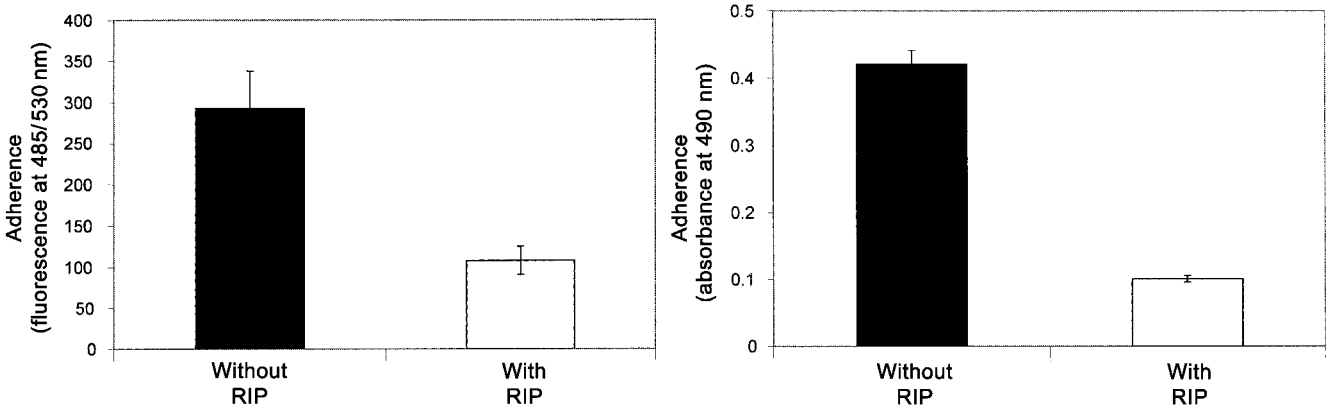

Figure 1. *A,* Reduction by RNAIII-inhibiting peptide (RIP) of *Staphylococcus epidermidis* adhesion to HaCat cells. Fluorescein isothiocyanate–labeled bacteria (10^6 cfu) were applied to microtiter plates containing a confluent layer of 10^4 HaCat cells, in the presence or absence of 5 μg of RIP. Cells were incubated for 30 min at 37°C and washed in PBS, and fluorescence at 485 and 530 nm was determined. *B,* RIP reduces adhesion of bacteria to plastic. *S. epidermidis* organisms were grown in polystyrene plates for 3 h. Adherent bacteria were stained by safranin, and the absorbance at 490 nm was determined.

Table 1. Prevention of *Staphylococcus epidermidis* infection with RNAIII-inhibiting peptide (RIP)–coated Dacron grafts in the presence or absence of local or parenteral antibiotic prophylaxis.

Strain, treatment	Bacterial concentration, mean cfu × 10^4/mL ± SD		
	GISE	MRSE	MSSE
Local prophylaxis			
Control (untreated)	680 ± 190	810 ± 220	730 ± 64
RIP	6.2 ± 2.4	0.74 ± 0.18	0.91 ± 0.23
Levofloxacin	280 ± 40	6.8 ± 2.5	4.2 ± 2.1
Levofloxacin + RIP	4.9 ± 0.79	0.26 ± 0.057	0.58 ± 0.072
Mupirocin	0.07 ± 0.018	0.035 ± 0.014	0.064 ± 0.015
Mupirocin + RIP	0	0	0
Rifampin	84 ± 27	0.8 ± 0.37	0.73 ± 0.2
Rifampin + RIP	5.9 ± 2.2	0.064 ± 0.013	0.031 ± 0.01
Quinupristin-dalfopristin	0.0069 ± 0.0013	0.0048 ± 0.001	0.0026 ± 0.0003
Quinupristin-dalfopristin + RIP	0	0	0
Parenteral prophylaxis			
Control (untreated)	680 ± 190	810 ± 220	730 ± 64
RIP	6.2 ± 2	0.74 ± 0.18	0.91 ± 0.23
Cefazolin	590 ± 200	370 ± 120	0.64 ± 0.19
Cefazolin + RIP	4.2 ± 1.6	0.35 ± 0.11	0.058 ± 0.034
Teicoplanin	57 ± 24	0.072 ± 0.008	0.0084 ± 0.0004
Teicoplanin + RIP	2.4 ± 1.8	0	0
Imipenem	89 ± 36	4 ± 1.8	0.73 ± 0.2
Imipenem + RIP	4 ± 2.5	0.088 ± 0.017	0.043 ± 0.012
Levofloxacin	480 ± 170	3.9 ± 1.4	3.5 ± 1.1
Levofloxacin + RIP	3.9 ± 3.3	0.29 ± 0.037	0.29 ± 0.055

NOTE. GISE, *S. epidermidis* with intermediate resistance to glycopeptides; MRSE, methicillin-resistant *S. epidermidis;* MSSE, methicillin-susceptible *S. epidermidis.*

catheter–related infections (exit site and tunnel) often are caused by *S. epidermidis* and can be difficult to eradicate (e.g., in patients undergoing hemodialysis). Tunnel infections almost always involve the catheter Dacron cuffs and are, therefore, more likely to lead to peritonitis; in this situation, catheter removal is the treatment of choice, because bacteria either evade antimicrobial treatment through biofilm formation or are resistant to many available antibiotics [3].

To test whether RIP can prevent graft-associated infections, Dacron grafts coated with RIP or without RIP and coated with various types of antibiotics or without antibiotics (for local prophylaxis experiments) were implanted in rats, and bacteria were injected into the implants. After 1 week, the implants were removed, and bacterial loads were determined. Alternatively, Dacron grafts were coated with RIP, bacteria were injected, and antibiotics were administered by the intraperitoneal route (for parenteral prophylaxis experiments). As a negative control, grafts were implanted without local or parenteral RIP/antibiotic prophylaxis, and no bacteria were injected. As a positive control, grafts were implanted and bacteria were injected, but no RIP/antibiotic prophylaxis was given. As a negative control for RIP, an inactive form of RIP analogue (YKPETNF) was used instead of RIP.

As shown in table 1, RIP reduced the bacterial load of all strains tested by log 3. When RIP is applied in conjunction with certain antibiotics, it can eliminate bacterial load by 100%.

None of the animals in the uncontaminated negative-control group had microbiologic evidence of graft infection, but all 15 rats in the contaminated, untreated positive-control group had evidence of graft infection. Quantitative culture results were $6.8 \times 10^6 \pm 1.9 \times 10^6$, $8.1 \times 10^6 \pm 2.2 \times 10^6$, and $7.3 \times 10^6 \pm 6.4 \times 10^5$ cfu/mL for the inoculated GISE, MRSE, and MSSE strains, respectively. Grafts coated with the inactive RIP analogue showed evidence of graft infection similar to untreated controls; quantitative culture results were $6.52 \times 10^6 \pm 3 \times 10^6$, $7.08 \times 10^6 \pm 2.1 \times 10^6$, and $5.4 \times 10^6 \pm 8 \times 10^5$ cfu/mL for the inoculated GISE, MRSE, and MSSE strains, respectively.

All groups with Dacron grafts soaked in 10 mg/L RIP showed evidence of decreased intensity of staphylococcal infection, compared with the untreated control group. Quantitative culture results were $6.2 \times 10^4 \pm 2.0 \times 10^4$ ($P < .05$), $7.4 \times 10^3 \pm 1.8 \times 10^3$ ($P < .001$), and $9.1 \times 10^3 \pm 2.3 \times 10^3$ cfu/mL ($P <$

.001) for GISE, MRSE, and MSSE strains, respectively. In rats inoculated with GISE, treatment of the graft with RIP inhibited bacteria better than prophylaxis with parenteral antibiotics (>10–100-fold; table 1) and was more effective than local prophylaxis with levofloxacin and rifampin but not as effective as local prophylaxis with mupirocin and quinupristin-dalfopristin (table 1). Among rats inoculated with MRSE or MSSE, RIP treatment was equally as effective as or more effective than most antibiotics, with the exception of teicoplanin, in the parenteral prophylaxis experiments, and mupirocin and quinupristin-dalfopristin, in the local prophylaxis experiments (table 1).

When treatment of the graft with RIP was accompanied by antibiotic prophylaxis, the level of inhibition of bacterial load was greater than that for single agents without RIP treatment, and, in some cases, the level of inhibition reached 100% (i.e., RIP treatment and parenteral administration of teicoplanin, for MRSE and MSSE strains [$P < .001$, vs. single agents] and RIP treatment and local administration of mupirocin or quinupristin-dalfopristin, for all strains [$P < .001$, vs. single agents]; table 1). To determine how much RIP bound to the Dacron graft, the graft was soaked in FITC-RIP, and fluorescence was determined. In these experiments, when 1 cm^2 of Dacron was soaked in a solution with 10 mg/L RIP, 26 μg of RIP bound to the Dacron. Of note, none of the agents used were associated with any toxicity, and no animals in any group died or had clinical evidence of drug-related adverse effects, such as local signs of perigraft inflammation, anorexia, vomiting, diarrhea, and behavior alterations.

Molecular mechanism of RIP: inhibition of TRAP phosphorylation. To test for the molecular mechanism of RIP, we carried out in vivo phosphorylation assays. The early exponential *S. epidermidis* strain Sofi was grown for 1 h in phosphate-free buffer with or without RIP (10 $\mu g/10^7$ cells), cells were collected, total cell homogenate was applied to SDS-PAGE gels, and gels were autoradiographed. As shown in figure 2, TRAP is phosphorylated both in *S. aureus* and in *S. epidermidis,* which suggests that TRAP may play an active role in regulating virulence in *S. epidermidis.* Moreover, RIP inhibits TRAP phosphorylation in *S. epidermidis* as it does in *S. aureus,* which suggests that RIP plays a similar inhibitory role in the pathogenesis of *S. epidermidis* and *S. aureus* infections.

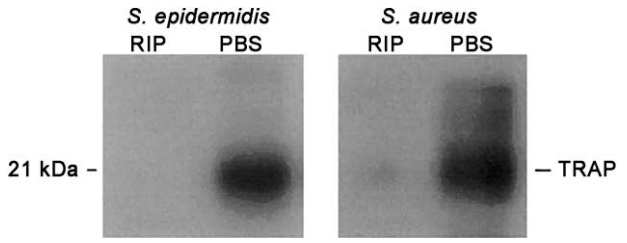

Figure 2. RNAIII-inhibiting peptide (RIP) inhibits target of RNAIII activating protein (TRAP) phosphorylation in *Staphylococcus aureus* and *S. epidermidis.* Both bacteria were phosphorylated in vivo for 1 h in the presence or absence of RIP. Total cell homogenate was separated by SDS-PAGE, and the gel was autoradiographed.

DISCUSSION

S. epidermidis virulence is often associated with the organism's ability to form a biofilm, which is regulated through a quorum-sensing mechanism [19, 26]. Our results show that adhesion and biofilm formation can be prevented both in vitro and in vivo by the quorum-sensing inhibitor RIP [23]. This heptapeptide prevents *S. aureus* infections by inhibiting the phosphorylation of TRAP, a 21-kDa protein that is unique to staphylococci and is highly conserved among staphylococcal strains and species [22]. The sequence of TRAP in *S. epidermidis* has 76% identity to that of TRAP in *S. aureus* (GenBank accession no. AF331164). This suggests that TRAP may play a regulatory role in various species of staphylococci and not only in *S. aureus.* Our results show that RIP inhibits TRAP phosphorylation in *S. epidermidis* as it does in *S. aureus,* which supports the idea that RIP action has a similar mechanism in *S. epidermidis.* Furthermore, our in vitro results show that RIP can influence adhesion of *S. epidermidis* on surfaces as living cells or plastic. Because adhesion is a major factor in *S. epidermis* virulence, RIP could be an effective inhibitor of *S. epidermidis* infections.

Peritoneal catheter-related infections (exit and tunnel) are predominantly caused by *S. epidermidis* and can be difficult to eradicate. Tunnel infections almost always involve the catheter Dacron cuffs and, therefore, are more likely to lead to peritonitis. In this situation, catheter removal is often required, because bacteria either evade antibiotic treatment through biofilm formation or are resistant to antibiotics. To prove the efficacy of RIP in preventing *S. epidermidis* adhesion and biofilm formation in vivo, a well-characterized experimental Dacron graft rat model [27–29] was used.

The collagen graft was soaked in RIP with or without antibiotics. RIP alone reduced bacterial load by log 3. When the graft was soaked in an inactive RIP analogue (a peptide that has no effect in vitro on cell adhesion or toxin production), no reduction in graft-associated infection was detected, which suggests that infection was specifically reduced because of RIP activity and not because of fortuitous peptide effect. To test whether RIP is synergistic with antibiotics, either grafts were coated with both RIP and antibiotics (local prophylaxis) or grafts were coated with RIP only and animals were injected with a single dose of antibiotics (parenteral prophylaxis).

Antibiotics from different families, including mupirocin, rifampin, glycopeptides (teicoplanin), cephalosporins (cefazolin), quinolones (levofloxacin), β-lactams (imipenem), and streptogramins (quinupristin-dalfopristin) were chosen because

they are currently used in clinical practice against staphylococci [30]. Some antibiotics were very effective in our experimental model, in particular, mupirocin, teicoplanin, and streptogramins (quinupristin-dalfopristin), as would be expected on the basis of results from other studies [30]. However, as with RIP alone, none of the antibiotics administered without RIP completely eradicated bacterial infection. Total (100%) inhibition was reached only when RIP was combined with mupirocin, teicoplanin, or a streptogramin (quinupristin-dalfopristin), which suggests that synergism exists between RIP and antibiotics.

The failure of RIP to cause total inhibition when used alone could be due to the actual amount of RIP that bound to the graft. It is estimated that soaking the graft in 10 mg/L RIP resulted in binding of 26 μg of RIP to the graft before implantation, which may not be enough to prevent adhesion of the 10^7 cells that were injected. Perhaps if the grafts had been coated with a higher concentration of RIP, better prevention could have been achieved. We must stress, however, that such a high level of a single-dose bacterial contamination is unlikely in clinical settings, and therefore the amount of RIP used during this in vivo experiment may be sufficient for precoating of grafts in clinical practice. Another hypothesis involves bacterial escape mechanisms that RIP cannot prevent. In this case, synergy with other antibiotics allows better treatment over a spectrum of bacterial virulence activities, reaching 100% inhibition when RIP is added to certain antibiotics (e.g., glycopeptides or streptogramins).

In conclusion, we propose an alternative approach to preventing infection and biofilm formation by use of drug-susceptible and -resistant *S. epidermidis* strains—the use of RIP, which modulates the bacterial mechanism of communication, inhibiting pathogenesis.

Acknowledgement

We thank I. Ofek for critical review of our manuscript.

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