Treatment of *Staphylococcus aureus* Biofilm infection by the quorum sensing inhibitor RIP

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Running title: Treatment of Staphylococcal infections by RIP

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ABSTRACT

Quorum-sensing inhibitor RIP inhibits Staphylococcal TRAP/agr systems and both TRAP– and agr– strains are deficient in biofilm formation in vivo, indicating importance of quorum-sensing to biofilms in the host. RIP injected systemically in rats treats methicillin-resistant S. aureus graft infections, suggesting that RIP can be used as a therapeutic.

NOTE

Millions of indwelling medical devices are implanted annually and are at risk of persistent infections caused by bacteria organized as a biofilm. Such biofilms are resistant to antibiotics, are difficult to treat, and are common causes of morbidity and mortality (1,14,17,26,29,13,34,35). Staphylococcus aureus and S. epidermidis are common causes or device-associated infections. S. aureus (like Pseudomonas aeruginosa), regulate virulence through two quorum-sensing (QS) systems that regulate one another (4,23,33,28,40). One QS uses the 33kDa autoinducer “RNAIII Activating Protein” (RAP) that induces the phosphorylation of “Target of RAP” (TRAP) (4,5,8,22,23,24,33). The other QS uses the autoinducing peptide AIP that phosphorylates AgrC, resulting in production of RNAIII and toxins (25,27,30,31).

TRAP is a 21kDa protein that was shown to regulate the expression of the many toxins and their regulator agr (9,23). In the absence of TRAP expression or phosphorylation, the bacteria do not express toxins and do not cause disease (tested in multiple strains and species of S. aureus and S. epidermidis) (9,22,39,41). To directly test biofilm formation by TRAP and agr mutants in vivo, TRAP– and agr– mutants and their parent strains were injected onto a graft (n=10) using the rat graft model. The bacteria used are S. aureus 8325-, a wild type (WT) lab strain and an isogenic derivative with an inactivated TRAP gene (TRAP–) (22), WT lab strain S. aureus RN6390, and its agr-null isogenic derivative RN6911 (30,31). Results show (Fig. 1) that TRAP– mutants formed very little biofilm (27±5 CFU/ml) as compared to
the control parent strain *S. aureus* 8325-4 (5.0x10^5 ± 1.1x10^5 CFU/ml) (p<0.0001) and that mutated *agr* (*S. aureus* RN6911) demonstrated reduced biofilm forming capacity (7.9x10^3 ± 1.3x10^3 CFU/ml) as compared to parent strain *S. aureus* RN6390 (4.8x10^5 ± 1.7x10^5 CFU/ml) (p<0.0001). These results indicate that TRAP regulates multiple genes important for biofilm formation *in vivo* in addition to those regulated by *agr* and further indicates the usefulness of TRAP as a therapy target-site.

TRAP phosphorylation can be inhibited by the quorum sensing inhibitor RNAIII Inhibiting Peptide (RIP). RIP (YSPWTNF-NH2) (21) has already been tested in multiple animal models with strong activity in preventing Staphylococcal infections including those caused by drug resistant strains like MRSA, GISA and VISA (2,3,5-7,9,11,12,15,16,19-21,36). No toxicity has been noted, and no resistant strains have emerged.

To test if RIP can also treat pre-formed device-associated Staphylococcal infections (biofilm), a graft rat model was used (legend Fig. 1). As a model for parenteral treatment, *S. aureus* strain Smith diffuse (SD) (5) was injected onto the graft. Animals (n=5) received intraperitoneal injections with various doses of RIP (0,10,20,30 mg/kg given every day for 1,4 or 7 days), administered immediately or starting two days after graft implantation. All grafts were explanted on day 10. Some of the grafts from control challenged but untreated animals were removed at day 3 to evaluate the biofilm. As shown in Fig. 2A, all control untreated animals demonstrated evidence of graft infection (6.9x10^7 ± 1.8x10^7 CFU/ml). In contrast, all rats included in the prophylaxis group (single dose of RIP injected immediately after implantation) showed concentration-dependent reduction in bacterial load, with 5.0x10^4 ± 2.1x10^4 CFU/ml if they were given 10mg/kg RIP, 9.4x10^3 ± 3.3x10^3 CFU/ml if given 20mg/kg RIP, and 2.3x10^3 ± 0.8x10^3 CFU/ml if given 30mg/kg RIP. RIP administered as a single dose, two days post implantation and bacterial challenge, also demonstrated reduction
of biofilm (Fig. 2A); rats administered a single dose of 10mg/kg RIP had \(8.4 \times 10^6 \pm 3.2 \times 10^6\) CFU/ml; rats administered a single dose of 20mg/kg RIP had \(3.0 \times 10^6 \pm 1.1 \times 10^6\) CFU/ml; and rats administered a single dose of 30mg/kg RIP had \(7.6 \times 10^5 \pm 2.8 \times 10^5\) CFU/ml. All results were significant (p<0.0001 with respect to controls).

RIP demonstrated notably greater treatment suppression of two-day old biofilms when administered in multiple doses. Treatment activity correlated with both dose and duration of treatment. As shown in Fig. 2B, rats administered four doses of 10mg/kg RIP had \(5.3 \times 10^4 \pm 1.9 \times 10^4\) CFU/ml; rats administered four doses of 20mg/kg RIP had \(6.3 \times 10^3 \pm 2.0 \times 10^3\) CFU/ml; and rats administered four doses of 30mg/kg RIP had \(8.5 \times 10^2 \pm 3.2 \times 10^2\) CFU/ml (p<0.0001 with respect to controls).

As also shown in Fig. 2B, rats given seven doses of 10mg/kg RIP had \(6.7 \times 10^3 \pm 1.7 \times 10^3\) CFU/ml, rats given seven doses of 20mg/kg RIP had \(4.1 \times 10^2 \pm 1.8 \times 10^2\) CFU/ml, and rats given seven doses of 30mg/kg RIP had \(2.9 \times 10^2 \pm 0.5 \times 10^2\) CFU/ml (p<0.0001 with respect to controls).

To test the synergistic effect of RIP and antibiotics, grafts were implanted, and animals (n=15) were challenged with MRSA ATCC43300. Three days after graft implantation and biofilm establishment, animals received intraperitoneal injections of RIP (10mg/kg) and/or Teicoplanin (3mg/kg) every day for 7 days. To evaluate treatment activity, grafts were explanted on day 10. As controls, grafts were explanted on day 3 and on day 10 to evaluate biofilm in untreated animals. As shown in Fig. 2C, \(3.8 \times 10^7 \pm 1.3 \times 10^7\) CFU/ml were present on the graft on day 3 and \(5.8 \times 10^8 \pm 1.8 \times 10^8\) CFU/ml were present on day 10, suggesting that a biofilm already existed on the day of treatment initiation. Similar results were obtained in grafts explanted on day 2 of biofilms formed by strain SD (not shown). As also shown in Fig. 2C, animals treated with seven doses of 10mg/kg RIP had \(7.8 \times 10^4 \pm 3.0 \times 10^4\) CFU/ml, animals treated with 3mg/kg Teicoplanin had \(3.6 \times 10^4 \pm 1.2 \times 10^4\) CFU/ml, and animals treated with
10mg/kg RIP + 3mg/kg Teicoplanin had $4.4 \times 10^2 \pm 1.6 \times 10^2$ CFU/ml. These results clearly demonstrate significant (p<0.0001 with respect to controls) reduction in bacterial load when animals were treated jointly with RIP and antibiotics.

From these data, we demonstrate that a biofilm is well-formed by the second day of graft infectivity, making it possible to treat the animal from day 2, excise the graft by day 10, and test whether or not treatment can enable biofilm eradication. Treatment of rats with RIP was most effective when administered in multiple doses, with suggestions of a dose and duration-dependent effect on biofilm load reduction. We also show that low-dose RIP treatment, which is combined with antibiotics like Teicoplanin, results in augmented activity relative to either agent alone. These results provide encouraging support for further evaluation of RIP as a therapeutic for device-associated infections, including those caused by drug resistant strains.

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FIGURE LEGENDS

Figure 1. Formation of a biofilm in vivo by TRAP− or agr− mutants was tested using the rat graft model, where 1cm² collagen-sealed Dacron grafts (AlbograftTM, Italy) were implanted subcutaneously into Wistar rats. Exponentially growing bacteria ($2 \times 10^7$) were inoculated onto the grafts, and grafts were removed 10 days later and bacterial load on grafts were determined and expressed as CFU/ml (minimal detection is 10CFU/ml). Comparisons of the results were performed by the analysis of variance (ANOVA) on the log-transformed data. Significance
was defined by a $P$ value $\leq 0.05$. The study was approved by the animal research ethics committee of the I.N.R.C.A. I.R.R.C.S., University of Ancona. Rats ($n=10$) were challenged with *S. aureus* RN6390 (WT), RN6911 (*agr*–), 8325-4 (WT) or TRAP– strains.

**Figure 2A.** Prevention and treatment of *S. aureus* infection by a single dose of RIP: Using the rat graft model, rats ($n=5$) were challenged with *S. aureus* strain Smith diffuse (SD) and injected parenterally with a single dose of RIP immediately or two days after bacterial challenge. Grafts were removed on day 10 and bacterial load on grafts was determined and expressed as CFU/ml.

**Figure 2B.** Treatment of *S. aureus* infection by multiple doses of RIP: Using the rat graft model, rats ($n=5$) were challenged with *S. aureus* strain Smith diffuse (SD) and injected parenterally two days later with multiple doses of RIP (every day for 1, 4 or 7 days). Grafts were removed on day 10 and bacterial load on grafts was determined and expressed as CFU/ml.

**Figure 2C.** Treatment of MRSA biofilm by RIP (10mg/kg) and/or Teicoplanin (3mg/kg). Using the rat graft model, rats ($n=15$) were challenged with *S. aureus* MRSA and treated 3 days later by seven doses of RIP and/or teicoplanin (administered every day for 7 days). Grafts were removed on day 10 and bacterial load on grafts were determined and expressed as CFU/ml. As a control (Cont), grafts of challenged and untreated animals were removed at days 3 or 10.

**REFERENCES**


peptide prevents graft-associated infections by antibiotic-resistant staphylococci.


Fig. 1.

![Graph showing bacterial CFU/ml for different strains](image)

Fig. 2A

![Bar graph showing single dose prevention or treatment](image)