Staphylococcal secretory inhibitor of platelet microbicidal protein is associated with prostatitis source

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This study reports the detection of an extracellular staphylococcal product, designated secretory inhibitor of platelet microbicidal protein (SIPMP), that causes local inhibition of the bactericidal action of platelet microbicidal protein (PMP) in the fluid phase. Urethral isolates of *Staphylococcus aureus* ($n = 24$) and coagulase-negative staphylococci (CNS) ($n = 47$) from patients with or without chronic bacterial prostatitis (CBP) were tested. SIPMP production was tested by inhibition of PMP bioactivity against *Bacillus subtilis* and was expressed as percentage inhibition of PMP bactericidal activity. The PMP susceptibility of staphylococcal strains was determined by exposing bacterial cells to serial dilutions of PMP. Staphylococci from patients without CBP produced SIPMP at levels of 10.3 ± 1.2 and $13.25 \pm 1.72\%$ for *S. aureus* and CNS, respectively. Strains isolated from men with CBP inhibited PMP-induced killing of *B. subtilis* by $23.38 \pm 4.2\%$ ($P < 0.05$) and $23.69 \pm 1.87\%$ ($P < 0.01$) for *S. aureus* and CNS, respectively. SIPMP production correlated with staphylococcal resistance to PMP ($r^2 = 0.6082$ and 0.7264 for *S. aureus* and CNS, respectively). SIPMP represents a hitherto unrecognized determinant of staphylococcal pathogenicity. These results suggest that SIPMP production is associated with the CBP source. Data from this study may have significant implications for the understanding of the pathogenesis of CBP.

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INTRODUCTION

A number of micro-organisms are able to infect the reproductive tract tissues in humans with serious consequences for reproductive function (Domingue & Hellstrom, 1998; Everaert *et al.*, 2003). A common result of microbial infection of the reproductive tract is prostatitis, a condition that may lead to transient or permanent infertility (Schoor, 2002; Everaert *et al.*, 2003). Bacterial infection of the prostate may occur as a result of ascending urethral infection or by reflux of infected urine into prostatic ducts emptying into the posterior urethra (Terai *et al.*, 2000). Other possible routes of prostatitis include invasion of bacteria by lymphogenous or haematogenous spread (Domingue & Hellstrom, 1998). Chronic bacterial prostatitis (CBP) is a subtle illness, characterized by the persistence of bacteria in the prostatic secretory system (Schoor, 2002; Everaert *et al.*, 2003). The most common causative agents of CBP are coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* (Lowentritt *et al.*, 1995; Domingue & Hellstrom, 1998; Ivanov, 2005). As it is difficult to establish precisely the significance of various

micro-organisms in the pathogenesis of CBP, it is imperative to delineate both microbial and host factors that contribute to its development (Domingue & Hellstrom, 1998; Schoor, 2002; Hua *et al.*, 2005).

The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently (Hancock & Chapple, 1999; Dürr & Peschel, 2002). Such peptides have also been found by several authors in human platelets and are designated β -lysins (Donaldson & Tew, 1977), thrombocidins (Krijgsveld *et al.*, 2000), thrombodefensins (Dankert, 1988) and platelet microbicidal protein (PMP) (Yeaman, 1997; Ivanov, 2005). These peptides are secreted at sites of infection and exert microbicidal activity against many pathogens, including Gram-positive cocci. Dankert *et al.* (1995) demonstrated that thrombodefensins are an important defence factor against viridans streptococcal endocarditis. Wu *et al.* (1994) showed that resistance *in vitro* of clinical staphylococcal and viridans group streptococcal strains to rabbit PMP correlated with the endovascular infectious source. The results of several studies (Dhawan *et al.*, 1997; Kupferwasser *et al.*, 2002) have shown that susceptibility to PMP is associated with staphylococcal endocarditis, and Kupferwasser *et al.* (1999) demonstrated that the staphylococcal multidrug-resistance gene *qacA* also mediates resistance to PMP *in vitro*.

Abbreviations: CBP, chronic bacterial prostatitis; CNS, coagulase-negative staphylococci; PMP, platelet microbicidal protein; SIPMP, secretory inhibitor of platelet microbicidal protein.

In a recent publication, we showed that resistance of urethral staphylococcal and enterococcal strains to human PMP *in vitro* correlated with the diagnosis of CBP (Ivanov, 2005). However, it is surprising that an extracellular bacterial product demonstrating this remarkable anti-PMP potential has not been described. The purpose of this study was to define the existence of an extracellular substance, designated secretory inhibitor of PMP (SIPMP), detectable in cell-free staphylococcal culture supernatants, that displays hitherto unrecognized PMP-consuming properties. In this paper, we also report the detection *in vitro* of SIPMP phenotypes of urethral staphylococcal isolates, along with a comparison with isolates from patients with or without CBP.

METHODS

Preparation and standardization of PMP. PMP was prepared as described previously (Ivanov, 2005). Briefly, healthy human platelet-rich plasma was obtained by low-speed centrifugation of 1 day-outdated platelets from the Blood Donor Department of the Orenburg Regional Medical Center. The platelet-rich plasma was then dispensed into polypropylene tubes and centrifuged again at 250 g for 30 min at 25 °C. The supernatants were removed and centrifuged at 1000 g for 30 min at 25 °C and the sedimented platelets were washed three times with Tyrode's buffer (138 mM NaCl, 3.6 mM KCl, 10 mM NaHCO₃, 0.4 mM NaH₂PO₄, 10 mM MgCl₂, 6 mM glucose, adjusted to pH 7.3 with phosphoric acid). The washed platelets were suspended in 5 vols ice-cold 30% acetic acid and stirred in melting ice for 24 h. The resulting extract was centrifuged at 10 000 g for 15 min and the PMP-rich supernatant was recovered. PMP preparations were stored at -20 °C and used for subsequent studies within 30 days. PMP bioactivity assays were performed with *Bacillus subtilis* ATCC 6633, an indicator organism highly sensitive to the bactericidal action of PMP (Donaldson & Tew, 1977). Exposure of 10⁸ washed human platelets ml⁻¹ to 5 ml ice-cold 30% acetic acid resulted in mean supernatant protein concentrations of ~70 mg ml⁻¹. The PMP susceptibility of bacterial strains was determined by exposing bacterial cells to serial dilutions of PMP. *B. subtilis* was grown in tryptic soy broth (TSB) at 37 °C for 18 h; organisms were harvested by centrifugation, washed twice in PBS (pH 7.2) and resuspended in PBS prior to use. Bacteria were diluted to 10⁴ c.f.u. ml⁻¹ in PBS. Serial dilutions of PMP were prepared in PBS and 900 µl PMP aliquots were transferred to low-protein-binding protein microtitre tubes (Costar Glass Works). To each of the tubes, 100 µl bacterial suspension was added. The tubes were incubated on a rotary shaker (300 r.p.m.) at 37 °C. After 1 h, aliquots of 200 µl were plated on blood agar plates. The microbicidal activity of PMP was assessed the next day after counting colonies on the agar plates and defined as the concentration of protein that retained ≥50% lethality for *B. subtilis* (Hirsch, 1960). The bioactivity of

such PMP preparations against *B. subtilis* ranged from 0.5 to 1.0 µg ml⁻¹. Control samples were found to possess no anti-*B. subtilis* bioactivity.

Detection of PMP resistance and SIPMP production. Well-characterized urethral isolates of *S. aureus* (n=24) and CNS (*Staphylococcus epidermidis*, n=27; *Staphylococcus hominis*, n=20) from patients with or without CBP were kindly provided by Serge Cherkasov (Orenburg State Medical Academy, Orenburg, Russia). The determination of patients and cases from which the isolates were initially obtained as CBP and non-CBP was made by the contributing investigator, using standard clinical parameters, prior to knowledge of an isolates SIPMP production and PMP susceptibility. The bacteria were cultured overnight in TSB and cell-free supernatants were obtained by centrifugation. Staphylococcal supernatants were sterilized by filtration through 0.45 µm pore size membranes (Millipore). Each culture supernatant (0.6 ml) (an equal volume of TSB was loaded in the control tubes) was combined with 0.3 ml PMP at 3.0 µg ml⁻¹ and incubated at 37 °C. After 1 h, 100 µl *B. subtilis* suspension at 10⁴ c.f.u. ml⁻¹ was added to each of the tubes. The tubes were incubated on a rotary shaker (300 r.p.m.) at 37 °C. After 1 h, aliquots of 200 µl were plated on blood agar plates. Colonies were counted after overnight incubation at 37 °C and the numbers of surviving micro-organisms were calculated. SIPMP production was expressed as the percentage inhibition of PMP bactericidal activity and calculated using the formula: percentage inhibition = (No - Nk1)/(Nk2 - Nk1) × 100, where No was the number of surviving *B. subtilis* cells in the presence of staphylococcal supernatant and PMP, Nk1 was the number of surviving *B. subtilis* cells in the presence of PMP alone, and Nk2 was the number of surviving *B. subtilis* cells in TSB.

The PMP susceptibility of bacterial strains was determined by exposing 2 × 10³ bacterial cells to serial dilutions of PMP from 5 µg ml⁻¹ to 15 µg ml⁻¹, as described by Yeaman *et al.* (1992). To define the proportion of strains considered PMP-susceptible and PMP-resistant, a survival of ≤50% of the initial inoculum at a PMP concentration of 5 µg ml⁻¹ was considered to be a relative PMP-susceptibility breakpoint, as in the data of Wu *et al.* (1994).

Statistical analyses were performed using Student's *t*-test. Correlation between SIPMP production and PMP resistance was calculated as *r*² values by the Pearson correlation calculation using Microsoft Excel. Values of *P* ≤ 0.05 and *r*² ≥ 0.5 were considered to be significant.

RESULTS AND DISCUSSION

Among the 24 *S. aureus* isolates studied, 13 urethral isolates were from patients with CBP, whilst 11 isolates were from patients without CBP (Table 1). Of the CBP strains tested, 10/13 (76.9%) were considered to be resistant to the bactericidal action of PMP compared with only 2/11 of the non-CBP isolates (18.2%, *P* < 0.001). Furthermore, 3/13

Table 1. PMP susceptibility and SIPMP production of urethral staphylococcus isolates

Organism	No. of PMP-resistant strains/total		No. of SIPMP-producing strains/total	
	Non-CBP	CBP	Non-CBP	CBP
<i>S. aureus</i>	2/11	10/13*	3/11	8/13*
CNS	1/18	27/29*	3/18	23/29*

**P* < 0.01.

Table 2. PMP susceptibility of non-CBP and CBP isolates

No. of organisms (non-CBP/CBP)	No. of PMP-resistant strains (non-CBP/CBP) with different concentrations of PMP ($\mu\text{g ml}^{-1}$)		
	≤ 5	10	≥ 15
<i>S. aureus</i> (11/13)	9/3	1/7	1/3
CNS (18/29)	17/2	1/11	0/16

(23.1 %) of CBP strains were resistant to $\geq 15 \mu\text{g PMP ml}^{-1}$ (Table 2).

Of the 47 urethral CNS isolates tested, 29 and 18 were from CBP and non-CBP cases, respectively. A significantly higher proportion of CBP strains of CNS (93.1 vs 5.6 %) was resistant to PMP compared with non-CBP strains (Table 1; $P < 0.001$). Moreover, all non-CBP strains exhibited either low-level resistance or susceptibility to $10 \mu\text{g PMP ml}^{-1}$. However, 16/29 CBP strains (55.2 %) were resistant to higher bactericidal concentrations of PMP ($\geq 15 \mu\text{g ml}^{-1}$).

Among the SIPMP-producing strains, organisms isolated from patients without CBP had mean SIPMP production levels of 10.3 ± 1.2 and 13.25 ± 1.72 % for *S. aureus* and CNS, respectively (Table 3). In contrast to bacteria isolated from the control group, strains isolated from men with CBP demonstrated more intensive inhibition of the bactericidal activity of PMP. The culture supernatants of *S. aureus* from the CBP group were significantly more active in decreasing levels of PMP-induced killing of *B. subtilis* (23.38 ± 4.2 %, $P < 0.05$). CBP strains of CNS demonstrated SIPMP production at a mean level of 23.69 ± 1.87 % ($P < 0.01$). SIPMP production correlated with staphylococcal resistance to PMP ($r^2 = 0.6082$ and 0.7264 for *S. aureus* and CNS, respectively).

At local sites of microbial infection, platelets, neutrophils and macrophages release large amounts of different bactericidal peptides (Dürr & Peschel, 2002). CBP is characterized by recurrent urinary tract infections and persistence of bacteria in the prostatic secretory system, despite the presence of multiple antibacterial peptides in prostatic fluid (Fair & Parrish, 1981; Com *et al.*, 2003). There is an urgent need to understand the virulence properties of bacteria that are associated with chronic infection of the prostate. Identifying such factor(s) would be helpful in devising effective treatment strategies.

In the present work, we detected an extracellular staphylococcal product with remarkable anti-PMP potential, which, to our knowledge, has not been described before. We anticipate that this substance represents a novel determinant of staphylococcal pathogenicity, as it probably causes local inhibition of the bactericidal action of PMP. As PMP may play an important role in the killing of staphylococci and preventing the onset of bacterial infection of the prostate, we speculated that SIPMP might enhance the infectivity of these bacteria through the abortive consumption of PMP in the fluid phase. The strategy underlying this process would be straightforward and effective. We believe that SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity. Similarly, in a study of the distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates by Hoe *et al.* (2001), 62 % of group A streptococci from patients with pharyngitis produced this extracellular protein. In addition, a recent study by Fernie-King *et al.* (2002) showed that purified secretory inhibitor of complement could block two additional components of the immune system: lysozyme and secretory leukocyte proteinase inhibitor. Collectively, our study and the results of several other studies (Bukharin *et al.*, 1996; Fernie-King *et al.*, 2002; Deckers *et al.*, 2004; Langley *et al.*, 2005) suggest that inactivation of components of innate immunity may be important for bacterial pathogens to induce and perpetuate chronic infections of different localization by surviving or avoiding microbicidal protein-mediated clearance.

Data from the present study may have significant implications for the understanding of the pathogenesis of CBP, as well as for future improvements in the prevention and therapy of CBP. Whether or not SIPMP is truly important in pathogenicity may become evident in the future from *in vivo* studies with selected SIPMP-positive and SIPMP-negative strains.

Table 3. SIPMP production of urethral staphylococcus isolates

No. of organisms (non-CBP/CBP)	No. of SIPMP-producing strains (non-CBP/CBP) with different levels (%) of SIPMP			
	0	0.1–10.0	10.1–20.0	> 20
<i>S. aureus</i> (11/13)	8/5	1/1	2/2	0/5
CNS (18/29)	15/6	1/3	2/5	0/15

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