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Direct and synergistic hemolysis caused by *Staphylococcus* phenol-soluble modulins: implications for diagnosis and pathogenesis

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Abstract

Phenol-soluble modulins are secreted staphylococcal peptides with an amphipathic α -helical structure. Some PSMs are strongly cytolytic toward human neutrophils and represent major virulence determinants during *S. aureus* skin and blood infection. However, capacities of PSMs to lyse human erythrocytes have not been investigated. Here, we demonstrate that many *Staphylococcus aureus* and *S. epidermidis* PSMs lyse human erythrocytes. Furthermore, synergism with *S. aureus* β -toxin considerably increased the hemolytic capacities of several PSMs. This synergism may be of key importance in PSM- and β -toxin producing *S. aureus* or in mixed-strain or -species infections with PSM and β -toxin producers. Of specific interest, several PSMs, in particular PSMA peptides, contributed to a considerable extent to synergistic hemolysis with β -toxin or when using the β -toxin producing strain RN4220 in CAMP assays. Thus, CAMP-type assays should not be used to detect or quantify *S. aureus* δ -toxin production, but may be used for an overall assessment of Agr functionality. Our study suggests an additional role of PSMs in staphylococcal pathogenesis and demonstrates that the repertoire of staphylococcal hemolysins is not limited to *S. aureus* and is much larger and diverse than previously thought.

Keywords

Staphylococcus aureus; *Staphylococcus epidermidis*; hemolysis; beta-toxin; delta-toxin; phenol soluble modulin

1. Introduction

Staphylococcus aureus is a dangerous human pathogen and one of the leading causes of infections worldwide [1]. The virulence potential of *S. aureus* is determined to a large extent by a multitude of secreted proteins that are toxic to humans. Many *S. aureus* toxins, such as superantigenic toxins or leukocyte-specific cytotoxins (leukocidins), interfere with key functions of acquired and innate host defense [2]. In addition, *S. aureus* produces a series of hemolysins [3]. Moreover, some *S. aureus* toxins may act in a synergistic fashion, of which the best-known example is the strong hemolysis exhibited by the synergism of β -toxin and

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δ -toxin [4]. *S. aureus* β -toxin is a sphingomyelinase that lyses erythrocytes [5], but also has leukotoxic properties [6]. The δ -toxin is a 26-amino acid peptide that damages membranes by pore formation [7]. This exemplifies how different mechanisms may work together to increase the capacity of *S. aureus* to harm target cells.

While *S. aureus* toxin production is highly strain-specific, because most toxins of *S. aureus* are encoded on mobile genetic elements, some selected toxins such as α -toxin and the recently discovered phenol-soluble modulins (PSMs) are encoded on the *S. aureus* core genome and produced by virtually all *S. aureus* strains. PSMs are a family of amphipathic α -helical peptides that include the δ -toxin [8, 9]. Members of the PSM family are present in most if not all staphylococci, particularly those that are pathogenic to humans [10]. The PSM repertoire is specific to a given staphylococcal species, often with only a limited degree of amino acid similarity to PSMs of other species. In *S. aureus* and *S. epidermidis*, all PSMs have been characterized on the gene and protein level [8, 9, 11–13].

PSMs contribute strongly to key aspects of staphylococcal virulence: Some PSMs have pronounced capacity to lyse human neutrophils [9], while others structure biofilms [14]. Rarely, PSMs may also kill other bacteria [15, 16]. Most likely, all these activities depend on the detergent-like character of PSMs that facilitates the disruption of hydrophobic interactions in biological membranes or between cell surface molecules. In addition, all PSMs are pro-inflammatory due to activation of the formyl peptide receptor 2 [17], causing neutrophil chemotaxis, priming, and cytokine release [9, 10].

While lysis of human neutrophils by PSMs has been investigated in detail [9], the interaction of PSMs with other human cells, in particular erythrocytes, is poorly understood. In the present study, we analyzed the hemolytic capacities of all *S. aureus* and *S. epidermidis* PSMs using human erythrocytes. Our results indicate an important additional mechanism by which many PSM peptides may contribute to virulence, both independently and in a synergistic fashion with β -toxin. Furthermore, we demonstrate that the commonly used synergistic hemolysis (CAMP) test using sheep blood agar plates does not primarily detect *S. aureus* δ -toxin, for which it has been used frequently, but a series of PSM peptides with sometimes considerable synergistic hemolysis capacities.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacteria were grown in tryptic soy broth (TSB). Strains *S. aureus* RN4220, RN6390, and 8325-4 are derivatives of strain NCTC8325 [18]. Strain 252 is a standard hospital-associated methicillin-resistant *S. aureus* (MRSA) strain [19]. Strains MW2 (pulsed field type USA400) and LAC (USA300) are clinically important community-associated MRSA strains [20, 21]. Isogenic *psm* mutant strains of strains MW2 and LAC used herein have been described previously [9]. Isogenic double and triple *psm* mutants in strain MW2 (MW2 $\Delta\alpha/\beta$; MW2 $\Delta\alpha/hld$; MW2 $\Delta\beta/hld$; MW2 $\Delta\alpha/\beta/hld$) were constructed for this study by an allelic replacement protocol as described previously [15]. Note that in “*hld*” mutants, only the start codon of the δ -toxin gene is mutated, abolishing translation, but RNAPIII functionality is maintained.

2.2. Peptides

All PSM peptides were synthesized by commercial vendors at > 95% purity with an N-terminal N-formyl methionine as found in naturally occurring PSMs.

2.3. Hemolysis tests

Human blood was obtained from healthy volunteers in accordance with a protocol approved by the Institutional Review Board for Human Subjects, NIAID. Informed written consent was received from human volunteers.

Hemolytic activities of synthetic PSM peptides at different concentrations were determined by incubating samples with human erythrocytes (2% v/v in Dulbecco's phosphate-buffered saline) for 1 h at 37°C as previously described [9]. For experiments comparing synergistic and direct hemolysis, and human versus sheep erythrocytes, purified β -toxin (β -toxin solution for CAMP test, Hardy Diagnostics) was applied to synergism samples at a dilution of 1:16 (sheep erythrocytes) or 1:4 (human erythrocytes). All samples were incubated at 37°C for 30 min. Hemolysis was determined by measuring OD_{540nm} using an ELISA reader.

2.4. Agar plate hemolysis tests

Sheep blood agar plates were prepared using defibrinated sheep blood (Becton Dickinson, 5% in TSB, 15 ml per plate) and used to analyze direct or synergistic hemolysis using bacterial cultures or filter disks with PSM solutions or culture filtrates. These were placed close to a filter with purified β -toxin solution, or a streaked culture of strain RN4220. Plates were incubated for 24 h before analysis.

2.5. Detection and quantification of PSMs

PSM peptides were quantified using reversed phase chromatography/electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) on an Agilent 1100 chromatography system coupled to a Trap SL mass spectrometer using a Zorbax SB-C8 2.3 × 30 mm column as previously described [11]. Peak areas were calculated using the two most abundant peaks of the electrospray ion mass spectra of the respective PSM peptides.

3. Results

2.1. Hemolytic activities of *S. aureus* and *S. epidermidis* PSMs

We first analyzed the capacities of all *S. aureus* and *S. epidermidis* PSMs to cause direct (β -toxin-independent) lysis of human erythrocytes. Hemolytic capacities of the analyzed PSMs differed strongly, with most pronounced hemolysis seen with *S. aureus* PSMs α 1, α 2, α 3, β 1, *S. epidermidis* PSM δ , *S. aureus* and *S. epidermidis* δ -toxins, and the SCC_{mec} encoded PSM-mec that is present in some methicillin-resistant strains of both species [13]. For some PSMs (e.g. *S. aureus* PSMs α 1, α 2, α 3), these results correlate with the capacities to lyse human neutrophils and sheep erythrocytes [9], but others (e.g. *S. aureus* PSM β 1, the δ -toxins, and PSM-mec) had higher cytolytic activities toward human erythrocytes than toward those other cell types (Fig. 1).

2.2. Synergistic hemolysis by *S. aureus* and *S. epidermidis* PSMs

To determine the capacities of PSMs for synergistic (β -toxin-dependent) hemolysis, we measured hemolysis with human blood and addition of pure β -toxin. We also measured synergistic hemolysis in the same way using sheep blood to detect potential species-specific differences and evaluate whether the frequent use of sheep blood as a substitute allows adequate analysis of the pathogenic potential of PSMs toward humans.

Addition of β -toxin and *S. aureus* PSMs α 1, α 2, α 3, *S. epidermidis* PSM δ , either δ -toxin, or PSM-mec caused a strongly hemolytic phenotype with human blood (Fig. 2). For some PSMs, such as the δ -toxins and PSM-mec, the strong potential to lyse human erythrocytes was almost entirely due to synergism, while in others such as in particular *S. aureus* PSMs

$\alpha 1$ and $\alpha 2$, there was no pronounced increase of the already high direct hemolysis potential by synergism with β -toxin. The hemolytic capacities of other PSMs, such as *S. aureus* PSMs $\alpha 3$ and $\beta 1$, and *S. epidermidis* PSM δ , were due to a considerable extent to both direct and synergistic hemolysis.

Most PSMs showed pronounced differences in their capacities to cause synergistic hemolysis of sheep versus human blood. Of note, the β -toxin concentration had to be raised 4 times in assays with human compared to those with sheep blood to detect a similar range of synergistic hemolysis, indicating that synergistic activity of PSMs and β -toxin is in general stronger toward sheep than human erythrocytes. In particular, *S. aureus* PSMs $\alpha 1$, $\alpha 2$, and $\alpha 3$ caused much stronger synergistic hemolysis of sheep than human blood. Interestingly, synergistic hemolysis capacities were strongly reduced in the processed PSM $\alpha 1$ and PSM $\alpha 2$ peptides (PSM $\alpha 1$ Δ MG and PSM $\alpha 2$ Δ MG), which were shown to be bactericidal toward *Streptococcus pyogenes* [15], while with human blood there was no significant difference between PSMs $\alpha 1$ and $\alpha 2$ and their processed derivatives.

2.3. PSM-dependent direct and synergistic hemolysis on sheep blood agar plates

The common test for synergistic hemolysis, also called CAMP test, uses sheep blood agar plates and purified *S. aureus* β -toxin. However, *S. aureus* strain RN4220, a strain derived by multiple mutagenesis procedures from the parent strain NCTC8325 [18], is often used in lieu of purified β -toxin. Synergistic hemolysis with the β -toxin-producing strain RN4220 is believed to depend upon complementation of δ -toxin by other cross-streaked strains or applied culture filtrates [22]. This assay has therefore frequently been used to detect and quantify the production of δ -toxin. As our results suggested that PSMs other than δ -toxin contribute significantly to synergistic hemolysis with β -toxin, we performed a series of experiments aimed to verify the basis of synergistic hemolysis on sheep blood agar plates, in particular when using strain RN4220.

First, we placed filters with *S. aureus* and *S. epidermidis* PSM solutions (15 μ l, 1 mg/ml) on sheep blood agar plates. Under those conditions, only PSM $\alpha 3$ of *S. aureus* and PSM α of *S. epidermidis* showed strong, and the δ -toxins of both species moderate synergistic hemolysis together with cross-streaked RN4220 bacteria (Fig. 3). Then, we used isogenic gene deletion mutants of the *S. aureus psm* loci to determine the contribution of *S. aureus* PSMs to direct and synergistic hemolysis on sheep blood agar plates, using bacterial cultures or culture filtrates and filters with pure β -toxin. Direct hemolysis of some *S. aureus* strains (for example, 8325-4 and LAC, Fig. 4A) on sheep blood agar plates can be very extensive, likely due to pronounced production of α -toxin. However, direct hemolysis in 24-h culture filtrates was entirely due to PSM α peptides in strain LAC, possibly because PSMs are more resistant to proteolytic degradation than α -toxin (Fig. 5). In contrast to strain LAC, strain MW2 did not show extensive direct hemolysis and synergism was easily detectable with that strain (Fig. 4A). Therefore, we used it for our synergism assays on sheep blood agar plates. Bacterial colonies of MW2 and a series of *psm* mutants with single, double, or simultaneous deletion of all known *psm* loci of *S. aureus* produced synergistic hemolysis phenotypes that indicated that all PSMs contribute to synergistic hemolysis on sheep blood agar plates (Fig. 4B,C). Furthermore, the *psm* triple mutant (MW2 $\Delta\alpha/\beta/hld$) did not cause any direct or synergistic hemolysis, demonstrating that those phenotypes are entirely due to PSMs in strain MW2.

Taken together, our results demonstrate that many PSMs cause strong synergistic hemolysis with β -toxin on sheep blood agar plates. As those capacities often reached that of δ -toxin, depending on the assay used, these findings also strongly suggest that the mechanistic basis of synergistic hemolysis with strain RN4220 is, at least in part, due to the production of PSMs in the test strains that are different from δ -toxin. Finally, results obtained with blood

agar plates did not reflect entirely those obtained using hemolysis assays in solution, indicating that additional factors – such as diffusion capacities – may impact the outcome of agar plate-based CAMP tests.

2.4. PSM production in the *S. aureus* NCTC8325 lineage

To further elaborate on the finding that PSMs other than δ -toxin may be more potent synergistic hemolysins when used in CAMP tests with strain RN4220, we analyzed PSM production in strains of the NCTC8325 lineage, in comparison to a series of other *S. aureus* strains. We found that all strains of the NCTC8325 lineage are characterized by a very low production of PSM α peptides compared to that of δ -toxin (Fig. 6). In contrast, production of δ -toxin in NCTC8325 was in the same range as in strains MW2 and LAC. Derivatives of the NCTC8325 strain (strains 8325-4, 6390, and RN4220) showed an even lower production of PSM α peptides than strain NCTC8325, close to the detection limit of our assay, while production of δ -toxin was only reduced by a factor of ~ 2 in strains RN4220 and RN6390 compared to NCTC8325. These findings further support the notion that synergistic hemolysis in the sheep blood agar test with RN4220 is not exclusively due to δ -toxin. Synergistic hemolysis caused by many *S. aureus* strains may rather depend on the production of PSM α or similar peptides that complement the absence of those peptides in strain RN4220.

4. Discussion

The investigation of PSM-dependent cytolysis has so far been focused on human neutrophils, owing to the key importance of neutrophils for the establishment of *S. aureus* infection. However, it is likely that the strong impact that PSMs have on *S. aureus* bacteremia, skin infection, and possibly other infection types, is caused by the interaction of PSMs with a variety of human cell types. Lysis of erythrocytes by PSMs in particular may play a key role for the progress of infection, but has only been investigated in a preliminary fashion using sheep blood. In this study, we therefore analyzed the interaction of PSMs with human erythrocytes.

As the β -toxin gene is non-functional in most *S. aureus* strains of clinical importance, due to insertion of the bacteriophage phi 13 [23], direct rather than synergistic hemolysis presumably plays a more important role for *S. aureus* pathogenesis. We found that direct hemolysis is very pronounced with the *S. aureus* PSM $\alpha 1$, $\alpha 2$, and $\alpha 3$ peptides. These findings highlight the key importance of the PSM α peptides as virulence determinants of *S. aureus* and indicate that they also influence pathogenesis by mechanisms that are not related to neutrophils.

The capacity to produce factors that show a positive reaction in the CAMP test, i.e. synergistic hemolysins, has been described for a multitude of staphylococcal strains and species [4]. However, these hemolysins have commonly been believed to represent homologues of the δ -toxin, with very few exceptions such as the slush peptides of *S. lugdunensis* [24]. Our results indicate that the capacity to cause synergistic hemolysis with β -toxin that is present in a multitude of *S. aureus* and *S. epidermidis* strains is to a large extent due to PSMs other than δ -toxin. This also suggests that the many yet uncharacterized PSM molecules detected recently in many staphylococcal species [10] may have similar capacities. Of note, in contrast to previous studies, we quantified the potential of PSMs to cause synergistic hemolysis, showing that in many PSMs this capacity frequently reaches that of δ -toxin.

While staphylococcal species other than *S. aureus* have not been shown to produce β -toxin, synergistic hemolysis by PSMs of those species may contribute to pathogenesis in a mixed

species infection with β -toxin-producing *S. aureus*, in a way similar to synergism with other CAMP factor-producing bacteria such as Group A and B streptococci [25, 26] or *Propionibacterium acnes* [27]. Notably, synergistic hemolysis between *P. acnes* and *S. aureus* has recently been suggested as a target for immunotherapy against *S. aureus* [28]. A similar situation may arise in a mixed infection of β -toxin and non- β -toxin, PSM- producing *S. aureus* strains. Of particular interest, synergistic hemolysis was very strong with PSM-mec, the only PSM located on a mobile genetic element. Acquisition of an SCC_{mec} type II, III, or VIII element that contains PSM-mec by a β -toxin-producing strain may thus result in a greatly increased hemolytic capacity. Such an element may also be acquired from *S. epidermidis*, emphasizing the importance of horizontal gene transfer for virulence.

Our results also have important further implications related to the use of diagnostic tests. First, we detected significant differences in PSM hemolytic capacities with human versus sheep blood, indicating no sheep blood assays should be used when assessing the potential of PSMs to contribute to human pathogenesis. Second, our findings indicate that the synergistic hemolysis (CAMP) test should not be used for the detection of δ -toxin, for which immunological or HPLC procedures are more appropriate, because they are more specific, albeit also more time-consuming. However, as all PSM peptides are under strict control of Agr [9, 11, 13], the CAMP test still appears appropriate to assay for functionality of the Agr system. (The necessity to test for Agr functionality is due to frequent spontaneous mutations that arise in that regulatory system and may result in the misinterpretation of gene regulatory effects [29–31].)

Finally, our results on PSM production in strain RN4220 are in accordance with the detected slipped mispairing mutation in AgrA of that strain [18]. This mutation has been reported not to abolish but only delay δ -toxin expression [18], which is in accordance with the only slightly reduced δ -toxin levels in stationary phase culture filtrates that we detected. In contrast, PSM α peptide production was extremely low even at that late point in growth. Thus, as AgrA controls the *psma* and *psm* β operons in the same direct fashion as the *agr* P2 and P3 (*hld*) promoters [32] [33] and would therefore affect PSM α production similarly to that of δ -toxin, this mutation is unlikely the cause of the absence of PSM α peptides in strain RN4220. Rather, the failure to produce PSM α peptides at considerable amounts appears to be a characteristic of the entire NCTC8325 lineage, whose genetic reason is unknown. In derivatives of NCTC8325, further decrease of PSM α production might be due to additional mutations that are unrelated to, but possibly also include the mispairing mutation in AgrA. Of note, these results indicate that while the lack of a direct hemolysis phenotype of strain RN4220 is very likely due to the absence of α -toxin, as pointed out by Traber et al. [18], the lack of synergistic hemolysis is due to the lack of PSM α peptides rather than δ -toxin.

In conclusion, our study identifies PSMs as a family of potent hemolysins with a putative key function in staphylococcal infections. The strong synergistic hemolysis seen with some PSMs may further contribute to virulence in strains that express β -toxin or in co-infections with β -toxin producers. It also calls for a re-evaluation of the applicability of CAMP assays for the detection of *S. aureus* δ -toxin.

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Fig. 1. Lysis of human erythrocytes by *S. aureus* and *S. epidermidis* PSMs

PSMs at the given concentrations were incubated with a 2% solution of defibrinated human blood for 1 h at 37°C. Hemolysis was measured by determining OD_{540nm} in an ELISA reader. *S. aureus* PSMs are grouped on the left, *S. epidermidis* PSMs on the right. PSM-mec is produced by some methicillin-resistant strains of both species. PSMα1 ΔMG and PSMα2 ΔMG are naturally occurring processed PSMα1, or PSMα2, respectively, in which the two N-terminal amino acids (MG) have been removed.

Fig. 2. Synergistic in comparison to direct lysis of human and sheep erythrocytes by *S. aureus* and *S. epidermidis* PSMs

PSMs at final concentrations of 0.1 or 1 µg/ml were incubated with a 2% solution of defibrinated human blood for 30 min at 37°C. Hemolysis was measured by determining OD_{540nm} in an ELISA reader. *S. aureus* PSMs are grouped on the left, *S. epidermidis* PSMs on the right. PSM-mec is produced by some methicillin-resistant strains of both species. PSMα1 ΔMG and PSMα2 ΔMG are naturally occurring processed PSMα1, or PSMα2, respectively, in which the two N-terminal amino acids (MG) have been removed. Dashed lines depict the level of OD_{540nm} in control samples with only buffer or only β-toxin, which showed no hemolytic capacity.

Fig. 3. CAMP assay with *S. aureus* RN4220 and pure PSM peptides of *S. aureus* and *S. epidermidis*

PSMs were applied to filter disks (15 μ l, 1 mg/ml), dried, and filters were placed next to streaked RN4220 bacteria on sheep blood agar plates. Plates were incubated for 24 h and photographed. Note that both direct and synergistic hemolysis is seen with *S. aureus* PSM α 3 (arrows).

Fig. 4. CAMP reaction of different *S. aureus* strains and MW2 *psm* mutants

Cultures of different *S. aureus* strains (A) or MW2 wild-type or *psm* mutant strains (B) were spotted next to filters with β -toxin solution. Plates were incubated for 24 h and photographed. Note direct and synergistic hemolysis zones, as shown for strain MW2 as example (arrows). (C) MW2 and MW2 *psm* mutants were cross-streaked with RN4220 for analysis of synergistic hemolysis.

Fig. 5. Hemolysis of culture filtrates of LAC *psm* mutants on sheep blood agar plates. 24-h culture filtrates of strain *S. aureus* LAC (USA300) (LAC wt) and isogenic *psm* mutants were applied to filter disks (15 μ l, undiluted). Plates were incubated for 24 h and photographed.

Fig. 6. PSM production in strains of the NCTC8325 lineage

PSM production was measured by RPHLC/ESI-MS in stationary phase (8-h) cultures of strains of the NCTC8325 (8325) lineage and other strains for comparison. Values for PSM α 3 (left y-axis) and δ -toxin (right y-axis) are shown; differences between strains regarding PSM α 1, α 2, and α 4 values were similar to those seen with PSM α 3.