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Staphylocidal Action of Thrombin-Induced Platelet Microbicidal Protein Is Not Solely Dependent on Transmembrane Potential

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Thrombin-induced platelet microbicidal protein (tPMP) is a small, cationic, antimicrobial peptide released from rabbit platelets when stimulated with thrombin. We studied the relationship between staphylococcal transmembrane potential ($\Delta\psi$) and tPMP staphylocidal activity. A genetically related pair of *Staphylococcus aureus* strains, 6850 and JB1, which differ in $\Delta\psi$ generation (−143 and −97 mV, respectively) were used. Mutant JB-1 was substantially less susceptible to tPMP than the parental strain, 6850. Menadione supplementation, which normalized the $\Delta\psi$ of strain JB-1, did not restore JB-1 tPMP susceptibility. These findings suggest that the staphylocidal activities of tPMP require factors other than or in addition to an intact $\Delta\psi$.

The platelet has been traditionally viewed as a key component in the induction and propagation of endovascular infection (22). In contrast, recent evidence from our laboratory suggests that platelets may play an important role in host defense against blood-borne pathogens. This antimicrobial function of platelets has been hypothesized to be due to secretion of an antimicrobial peptide, termed thrombin-induced platelet microbicidal protein (tPMP) (30). tPMP is microbicidal against common bloodstream pathogens, including *Staphylococcus aureus*, viridans streptococci, and *Candida albicans* (25–27, 29, 30). However, the mechanism of tPMP microbicidal activity has not been fully defined. Several recent observations in our laboratory indicate that the staphylococcal membrane is the likely target for tPMP. First, flow cytometric data suggested that one of the known PMPs (PMP-2) permeabilizes the staphylococcal membrane in vitro (28). In addition, ultrastructural studies revealed that tPMP induces rapid and extensive staphylococcal cell membrane damage and death, followed by eventual cell lysis (30a). Several other cationic microbicidal molecules (e.g., aminoglycosides and some lantibiotics [lanthionine-containing antibiotics]) require a threshold bacterial transmembrane potential ($\Delta\psi$) to effect bactericidal activity (14, 21). Thus, the aim of the current study was to define the relationship between *S. aureus* $\Delta\psi$ and the staphylocidal activity of tPMP.

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S. aureus strains 6850 and JB-1 have been described previously (1). JB-1 is a respiratory-deficient, menadione-auxotrophic mutant of parental strain 6850. Because of its slow growth rate, aminoglycoside resistance, and microcolony morphology, strain JB-1 is considered a typical small-colony variant (1, 15). *Bacillus subtilis* 6633 was obtained from the American Type Culture Collection. *Staphylococcus simulans* 22 (previously known as *Staphylococcus cohnii* 22) was described previously (19).

tPMP was prepared by stimulating washed rabbit platelets (10^8 CFU/ml) with thrombin as described previously (30). The bactericidal activity of tPMP-rich preparations was determined by bioassay, as previously described, with *B. subtilis* ATCC 6633 used as a highly tPMP-sensitive indicator organism (28).

Gentamicin E-strips were obtained from AB Biodisk (Piscataway, N.J.). The lanthionine-containing cationic antibiotics Pep5 and epidermin were isolated from *Staphylococcus epidermidis* strains 5 and 57, respectively, and purified by high-pressure liquid chromatography as described elsewhere (7, 19). Three other lantibiotics, mersacidin, cinnamycin, and nisin, were kindly provided by Hoechst Aktiengesellschaft (Frankfurt, Germany), G. Jung (University of Tübingen, Germany), and Aplin & Barrett Ltd. (Dorset, England)/Koch & Light (Colnbrock, England), respectively. The human neutrophil defensin HNP-1 was generously provided by M. E. Selsted (University of California, Irvine). The MICs of gentamicin against strains 6850 and JB-1 were determined by using E-strips, according to the protocol described by the manufacturer, at a final inoculum of 4×10^6 CFU per plate. MICs were read directly from the E-strips as the value where the inhibition zone edge intersected the E-strip scale. The MICs of the cationic lantibiotics, nisin, Pep5, epidermin, mersacidin, and cinnamycin were determined by standard Trypticase soy broth dilution assays in microtiter plates (final volume, 500 μl; final lantibiotic concentration range, 0.0003 to 76.8 μg/ml; final bacterial inoculum, 10^4 CFU/ml). The microtiter plates were incubated at 37°C for either 16 to 18 h (strain 6850) or 48 h (strain JB-1). MICs were defined as the minimum lantibiotic concentration which prevented visible staphylococcal growth. *S. simulans* 22, a routine indicator strain for Pep5, was used as a positive control for lantibiotic activity in all MIC determinations (19). All MIC assays were repeated at least twice on separate days and were highly reproducible.

Nonspecific binding and reduction of tPMP and HNP-1 antimicrobial activities occur in nutrient medium. As a result, timed bactericidal assays in defined buffer solutions were used

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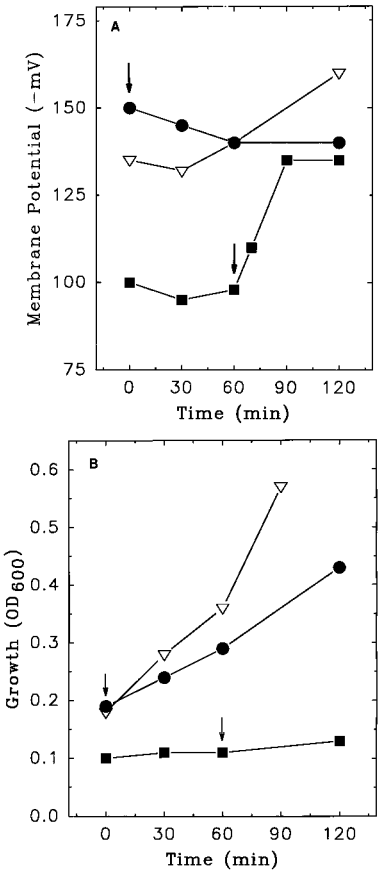

FIG. 1. Effect of menadione on transmembrane potential and growth of *S. aureus* 6850 and JB-1. (A) The transmembrane potential of strain 6850 (▽) was measured in the absence of menadione during logarithmic growth. Measurements for strain JB-1 were obtained from cultures that were either pregrown (●) or pulsed (■) with menadione (1 μg/ml; time of addition is indicated by arrows). (B) Growth of cultures was determined concurrently with Δψ measurements. ▽, strain 6850, no menadione; ●, strain JB-1, with menadione; ■, strain JB-1, pulsed with menadione. The time of menadione addition is indicated by arrows. Data represent means of two experiments with low variability.

to determine the susceptibilities of logarithmic-phase cells of 6850 and JB-1 to tPMP and HNP-1. Nisin was also included in the microbicidal assays as a control cationic peptide. The following incubation buffers, found in pilot studies to support bacterial viability and permit maximum bactericidal activity of the respective cationic peptides, were used: Eagle's minimal essential medium (MEM; Irvine Scientific, Santa Ana, Calif.), pH 7.4 (tPMP and nisin [in darkness]) and Hanks' balanced salt solution (HBSS; Irvine Scientific), pH 7.4 (HNP-1). Strains 6850 and JB-1 (10^5 CFU/ml) were exposed to the following final peptide concentrations: 0.5 μg/ml for tPMP; 5, 15, 25, and 40 μg/ml for HNP-1; and 50 μg/ml for nisin. The surviving bacterial population was enumerated by quantitative culture on blood agar plates after incubation at 37°C for 2 h. Staphylococcal susceptibilities to tPMP and HNP-1 were then plotted against peptide concentration, while nisin was plotted against exposure time. All experiments were performed in triplicate on separate days, and the data recorded represent mean values ± standard error.

S. aureus JB-1 is a menadione-auxotrophic, small-colony variant of strain 6850 (1). Therefore, the influence of menadione supplementation on the in vitro susceptibility of strain JB-1 to cationic antimicrobial agents was determined. MIC and microbicidal assays were carried out as described above, with the addition of menadione (1 μg/ml, in darkness), as follows: (i) for MIC assays, menadione was added prior to incubation at 37°C; (ii) for microbicidal assays, menadione was added at the beginning of bacterial growth to logarithmic phase; the logarithmic-phase cells were then washed and exposed to the various microbicidal peptides in the presence of menadione. At 1 μg/ml, menadione had no significant effect on cell viability (data not shown).

Values of Δψ were quantified by measuring radiolabeled tetraphenylphosphonium ($[^{14}C]TPP^+$) uptake during logarithmic growth for 2 h, as described elsewhere (21). TPP^+ is a lipophilic cation which diffuses across the bacterial membrane in response to a trans-negative Δψ (intracellular, negatively charged). Nonspecific $[^{14}C]TPP^+$ binding to bacterial cells was corrected by subtracting the radioactivity of 10% butanol-killed cells. A mean Δψ was calculated from a minimum of two independent determinations.

S. aureus 6850 exhibited a mean Δψ of −143 mV (Fig. 1A). In contrast, strain JB-1 had a mean Δψ of −97 mV in the absence of menadione (Fig. 1A). The addition of menadione to logarithmic-phase JB-1 cells resulted in an increase in Δψ to parental values, with a mean of −145 mV (Fig. 1A). Comparison of staphylococcal growth kinetics during Δψ measurements indicated that growth rates for strains 6850 and JB-1 correlated with the relative membrane Δψ values (Fig. 1B). In

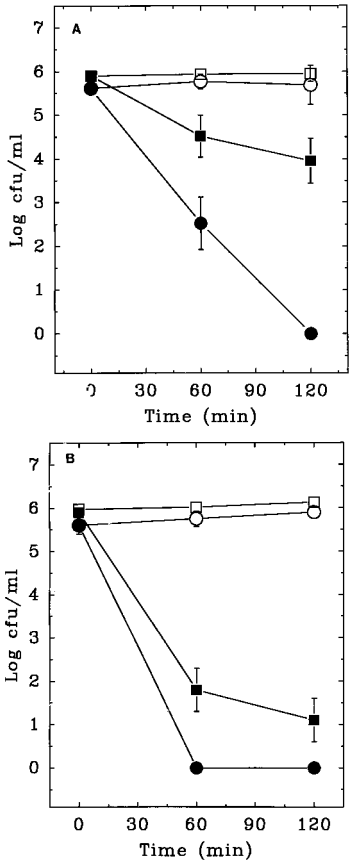

FIG. 2. Susceptibility of *S. aureus* 6850 and JB-1 to nisin. *S. aureus* strains 6850 (○, ●) and JB-1 (□, ■) were exposed for 2 h to either MEM buffer, pH 7.4 (○, □), or nisin (50 μg/ml, in darkness) (●, ■). Bacterial survival was enumerated throughout incubation on solid medium. Cells were grown (A) without menadione or (B) in the presence of menadione (1 μg/ml).

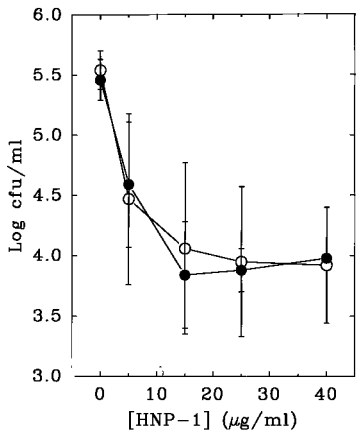

FIG. 3. Susceptibility of *S. aureus* 6850 and JB-1 to HNP-1. Cells of 6850 (○) and JB-1 (●) were exposed to various concentrations of HNP-1 for 2 h in HBSS buffer, pH 7.4. Survivors were enumerated on solid medium.

the absence of menadione, strain JB-1 grew more slowly than 6850. The addition of menadione to the slow-growing mutant culture at 60 min of incubation did not effect growth rate in the subsequent 60 min of incubation (Fig. 1B). In contrast, addition of menadione at the onset of growth resulted in the growth kinetics of JB-1 being similar to those of strain 6850 (Fig. 1B).

The broth dilution MICs for strains 6850 and JB-1 with a panel of cationic antimicrobial agents in the presence and absence of menadione are summarized in Table 1. Menadione restored nisin and gentamicin MICs for strain JB-1 to parental and near-parental levels, respectively. Similarly, timed bactericidal assays with nisin at 50 μg/ml revealed that strain JB-1 was substantially less susceptible to this peptide than strain 6850 (Fig. 2A). However, menadione supplementation resulted in a marked increase in JB-1 susceptibility to nisin, restoring it to near-parental levels (Fig. 2B). In the presence of menadione, the MIC of Pep5 for strain JB-1 was lowered twofold (Table 1). The MIC of Pep5 against *S. simulans* 22 (a known Pep5-susceptible strain) was 0.6 ng/ml, consistent with previous observations (3). Strains 6850 and JB-1 were equally susceptible to epidermin, mersacidin, and cinnamycin; in the presence of menadione, no alterations in MICs were observed for either strain (Table 1). Moreover, strains 6850 and JB-1 were equally highly susceptible to defensin HNP-1 across the concentration range tested (5 to 40 μg/ml) (Fig. 3).

S. aureus JB-1 was significantly less susceptible to tPMP than strain 6850 (Fig. 4A). The staphylocidal activity of tPMP against strains 6850 and JB-1 was tPMP concentration dependent but inoculum independent (data not shown). Importantly, the susceptibility of strain JB-1 to tPMP was not significantly altered by menadione supplementation (Fig. 4B).

The microbicidal action of many cationic molecules requires target organism generation of a threshold $\Delta\psi$ across the cytoplasmic membrane (13, 14, 21, 23). It is believed that the $\Delta\psi$ across the target membrane facilitates interaction between the cationic antimicrobial molecule and the bacterial phospholipid membrane, resulting in membrane disruption (6). tPMP, recently isolated from rabbit platelets, is cationic and has potent microbicidal activity against the common bloodstream pathogens (25–27, 29, 30). Strain JB-1, a well-described small-colony variant, is auxotrophic for menadione (1). Exogenous menadione serves as a biosynthetic precursor of menaquinone and can be isoprenylated for incorporation into the *S. aureus* electron transport chain as the initial electron acceptor (10). The failure of *S. aureus* to synthesize or incorporate menaquinone into the electron transport chain results in a cascade of secondary phenotypic abnormalities related to defective ATP generation (16, 17). Such abnormalities include slow growth rate, lack of hemolysis on blood agar, and microcolony phenotype, as observed in strain JB-1. Furthermore, ATP generation is required for $\Delta\psi$ maintenance. Thus, it was not unexpected that the $\Delta\psi$ of strain JB-1 (mean, −97 mV) was substantially lower than that of its genetically related parental strain 6850 (mean, −143 mV). The $\Delta\psi$ of strain JB-1 was restored to the parental level by menadione supplementation. This menadione-mediated resuscitation of JB-1's $\Delta\psi$ provided an important tool to investigate the influence of $\Delta\psi$ on the staphylocidal action of tPMP versus a panel of other cationic antimicrobial agents.

As mentioned above, aminoglycosides such as gentamicin require a threshold $\Delta\psi$ of about −95 mV for staphylocidal activity (14). As predicted, strain JB-1 was threefold less susceptible to gentamicin than strain 6850. The gentamicin susceptibility of this mutant was restored to a near-parental level by menadione supplementation.

Lantibiotics are a family of lanthionine-containing antibacterial peptides produced by gram-positive bacteria (for reviews, see references 4 and 20). These peptides are subdivided

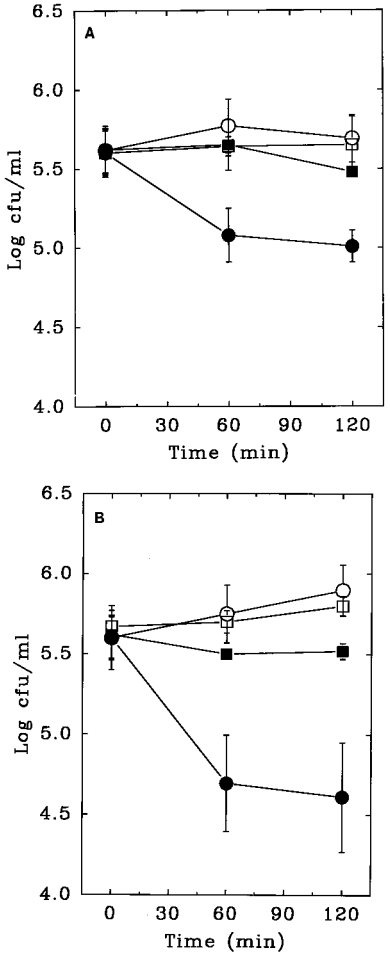

FIG. 4. Susceptibility of *S. aureus* 6850 and JB-1 to tPMP. Cells of 6850 (○, ●) and JB-1 (□, ■) were exposed to either MEM, pH 7.4 (○, □), or tPMP (0.5 μg/ml) (●, ■) for 2 h. Bacterial survival was enumerated throughout the incubation on solid medium and then plotted against time. Cells were grown (A) without menadione or (B) in the presence of menadione (1 μg/ml).

TABLE 1. Influence of menadione on *S. aureus* susceptibility to lantibiotics and gentamicin[a]

Cationic agent	MIC (μg/ml)			
	− Menadione		+ Menadione	
	SA 6850	JB-1	SA 6850	JB-1
Nisin	4.8	9.6	ND[b]	4.8
Pep5	1.2	9.6	ND	4.8
Epidermin	2.4	2.4	ND	2.4
Mersacidin	9.6	9.6	ND	9.6
Cinnamycin	75	75	75	75
Gentamicin	2	6	2	3

[a] All assays were carried out in Trypticase soy broth. Menadione was added at 1 μg/ml. Values indicate mean MICs from a minimum of two experiments.
[b] ND, not determined.

into two groups (A and B) based on their charge, size, and molecular structure. Type A lantibiotics, such as nisin, Pep5, and epidermin, are cationic and form Δψ-dependent pores in target bacterial membranes (11). Nisin and Pep5 require threshold Δψ values of −80 and −100 mV, respectively (values encompassing the Δψ generated by strain JB-1) (13, 21). In contrast, epidermin requires a Δψ of only −50 mV for bactericidal action (2, 18). The data obtained in this current study are consistent with published results on threshold Δψ and type A lantibiotic bactericidal activity. In MIC assays, strain JB-1 was less susceptible to nisin and Pep5 than strain 6850. The susceptibility of strain JB-1 to both nisin and Pep5 was restored to parental and near-parental levels, respectively, by menadione supplementation. Timed bactericidal assays of nisin produced data which are consistent with those from the MIC assays. In the former assays, menadione supplementation restored the nisin susceptibility of JB-1 to near-parental levels. In contrast to nisin and Pep5, strains 6850 and JB-1 were equally susceptible to epidermin, as predicted by its threshold Δψ of −50 mV. Type B lantibiotics (cinnamycin and mersacidin) inhibit phospholipases and cell wall synthesis, respectively (5), and do not require Δψ for microbicidal activity. As anticipated, strains JB-1 and 6850 were equally susceptible to these lantibiotics, reflecting the Δψ independence of their bactericidal activities.

Defensin HNP-1 is an endogenous, cationic microbicidal peptide stored in human neutrophils which appears to target the microbial cell membrane as its site of action (24) (for a review, see reference 8). Strains 6850 and JB-1 were equally susceptible to HNP-1. These findings suggest either that the staphylocidal activity of HNP-1 is Δψ independent or that the threshold Δψ needed for HNP-1 microbicidal activity is below the −97 mV generated by strain JB-1.

In comparison, strain JB-1 was substantially less susceptible to tPMP than strain 6850. This indicates that tPMP staphylocidal activity is related to a threshold staphylococcal Δψ. However, in contrast to gentamicin, nisin, and Pep5, the susceptibility of JB-1 to tPMP was not restored to the parental level by menadione supplementation. These results suggest that the staphylocidal mechanism of tPMP may be dependent on factors other than or in addition to Δψ. Of note, certain cationic peptides (e.g., nisin) depend on the net proton motive force (namely, Δψ and ΔpH) for maximal activity stimulation (9). Recent preliminary data from our laboratory indicate that external pH may also be important for tPMP staphylocidal activity (12, 12a). Studies are in progress to further investigate the relationship between the net bacterial proton motive force and tPMP staphylocidal activity by examining the effects of differing pH conditions as well as specific Δψ dissipators (e.g., valinomycin [14]).

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