INFECTION AND IMMUNITY, Aug. 1994, p. 3416–3423
0019-9567/94/$04.00+0

Vol. 62, No. 8

Platelet Microbicidal Protein Alone and in Combination with Antibiotics Reduces *Staphylococcus aureus* Adherence to Platelets In Vitro

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Received 10 January 1994/Returned for modification 5 April 1994/Accepted 16 May 1994

Bacterial adherence to platelets on the cardiac valve surface is believed to be critical in the induction of infective endocarditis. Recent studies have confirmed that thrombin-activated platelets secrete platelet microbicidal protein (PMP), which can both kill and exert nonlethal antiadherence effects against endovascular pathogens. In the present study, we quantified the influence of antibiotic and/or PMP exposures on in vitro platelet adherence of two *Staphylococcus aureus* strains, identical by DNA restriction and cell wall protein profiles, that differed in their susceptibility to PMP-induced killing (PMP^s or PMP^r, respectively). Adherence assays were performed by flow cytometry in the presence of sublethal PMP concentrations (1 to 2.5 μg/ml) alone or in combination with ampicillin (AMP) alone, sulbactam (SUL) alone, or AMP plus SUL (AMP-SUL), at levels achievable in serum. Exposure of the PMP^s and PMP^r *S. aureus* strains to antibiotics (for 2 h at 37°C) prior to flow cytometry resulted in no substantive changes in the percent adherence to platelets compared with that for *S. aureus* cells not exposed to antibiotics, except for modestly increased adherence of both PMP^s and PMP^r cells exposed to AMP-SUL (18.5 and 15.8% increases, respectively). Addition of PMP to antibiotic-*S. aureus* mixtures (final 30 min) caused a significant decrease in *S. aureus* adherence to platelets, for both the PMP^s and PMP^r *S. aureus* strains, compared with antibiotic exposure alone (e.g., reduction in platelet adherence from 57.9 ± 8.2% to 12.2 ± 3.6% for PMP^s cells exposed to AMP-SUL and PMP [$P = 0.01$]). Moreover, addition of PMP following exposure of the PMP^s and PMP^r strains to AMP-SUL reversed the enhanced bacterium-platelet adherence observed with such antibiotic exposures alone ($P \leq 0.005$). These data demonstrate that PMP exerts a potent antiplatelet adherence effect which is independent of its microbicidal capacity, rendering *S. aureus* cells less adherent to platelets in the presence or absence of antibiotics. Reduction of microbial adherence to platelets by PMP alone or with antibiotics provides further insight into the mechanism(s) that may be involved in host defense and antibiotic prophylaxis of infective endocarditis and other endovascular infections.

Endovascular infection may be defined as the localized presence of microorganisms adherent to or within the endocardium or vascular endothelium. Infections encompassed by such a definition include native and prosthetic valve infective endocarditis, mycotic aneurism, and endovascular catheter-related infections.

The mechanism(s) by which microbial pathogens, such as *Staphylococcus aureus*, induce endovascular infection is undoubtedly complex and multifactorial, involving the adhesive interactions of these organisms with host plasma proteins, endothelial cells, and platelets (7–10, 21). Such interactions are believed to be crucial in initiating the sequence of pathogenic steps required for an organism to establish endovascular infection. In this regard, platelets have traditionally been thought to promote induction of endovascular infection (e.g., infective endocarditis) and hematogenous dissemination of *S. aureus* by providing the organism with an adherence surface on damaged endothelium, facilitating evolution and maturation of vegetations (8, 21).

In contrast, recent evidence suggests that platelets may also serve an important host defense function against the induction and propagation of endovascular infection, via thrombin-induced α-granule release of platelet microbicidal protein (PMP) (27–33). Our laboratory and others have previously demonstrated that PMP exerts a potent microbicidal effect against common endovascular pathogens (e.g., coagulase-positive and coagulase-negative staphylococci, viridans group streptococci, and *Candida albicans* [26, 27, 30]); also, separate studies have demonstrated staphylocidal synergy of PMP in combination with antibiotics (29), suggesting that PMP and antibiotics interact favorably to kill bacteria in vivo. In addition to their microbicidal actions, PMP and antifungal agents have been shown individually and in combination to reduce *C. albicans* adherence to endothelial cells and platelets in vitro (13, 32), while antibiotics and PMP individually reduce bacterial adherence to the same cells (2, 22, 33). From this perspective, it is also possible that PMP and antibiotics in combination significantly alter bacterial adherence to platelets.

Further investigations into the effects of PMP and antibiotics

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in combination upon bacterium-platelet adherence may also clarify the operative mechanisms accounting for antibiotic prophylaxis of infective endocarditis. Prolonged growth inhibition by antimicrobial agents had been previously shown to be pivotal in this regard, presumably by rendering microbial pathogens more susceptible to neutrophil phagocytosis and killing (2, 3, 17, 18). However, recent investigations have demonstrated in vivo that antibiotic prophylaxis of infective endocarditis is equally effective in neutropenic and nonneutropenic animals (3). These findings implicated other host defense mechanisms, including platelets, as critically important components acting in conjunction with antibiotics to achieve successful prophylaxis of endovascular infection (11). Thus, we designed the current study to examine the abilities of PMP, alone and in combination with the antibacterial agents ampicillin (AMP) and sulbactam (SUL), to influence *S. aureus* adherence to platelets in vitro.

(This study was presented in part at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, October 1993 [31].)

MATERIALS AND METHODS

Preparation of PMP. PMP was prepared as described previously (27, 30). In brief, whole blood from healthy New Zealand White rabbits was freshly collected into polypropylene containers containing buffered citrate anticoagulant (anticoagulant-to-blood ratio, 1:5). Centrifugation (100 × *g* for 15 min at 25°C) produced an upper platelet-rich plasma suspension. Collection of the upper two-thirds of this platelet-rich plasma fraction routinely yielded platelets with <1% leukocyte contamination. Platelets were pelleted by centrifugation (2,000 × *g* for 10 min) of the upper two-thirds of the platelet-rich plasma fraction, and the resulting platelet pellet was washed twice in Tyrode's solution (pH 7.4, Sigma Chemical Co.). Washed platelets were then suspended to a concentration of 10^9/ml in prewarmed (37°C) Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.). Preparations rich in PMP were produced from such washed platelet suspensions by stimulation with bovine thrombin (1 U/ml; Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C in the presence of 0.2 M $CaCl_2$. Residual platelet material was subsequently removed by centrifugation (3,000 × *g* for 15 min), and the PMP-rich supernatant was recovered. PMP was then further purified by anionic affinity membrane adsorption and 1.5 M NaCl elution, as previously described (27, 30). Approximately 1 to 2 mg of substantially purified PMP was routinely recovered from 5 × 10^{11} platelets. Final PMP preparations were pooled, dialyzed against 10 volumes of sterile deionized water in tubing with a molecular mass cutoff of 3.5 kDa (Spectrapor 3; Spectrum Medical Industries, Los Angeles, Calif.), and stored at −20°C until use. PMP preparations stored in this manner exhibited no loss in antimicrobial activity after 6 to 12 months.

Bactericidal activity of the PMP preparation was established by using a modified bioassay in which *Bacillus subtilis* ATCC 6633 served as a highly PMP-sensitive indicator organism (30). PMP bioactivity was quantified as units per milliliter, in which the number of units represents the reciprocal of the highest PMP dilution yielding ≥95% killing of the *B. subtilis* inoculum (10^3CFU/ml) after a 30-min incubation (30). Specific PMP bioactivity was then determined as units per milligram of protein and converted to PMP concentrations expressed in micrograms per milliliter.

Organisms. *S. aureus* SA-19, a clinical methicillin- and oxacillin-resistant bloodstream isolate obtained from the Microbiology Laboratory of Harbor UCLA Medical Center, was used in these studies. This organism was selected because of its high susceptibility to the bactericidal action of PMP (PMP^s; ≤10% survival of a 2 × 10^3-CFU/ml inoculum following exposure to PMP at 100 U/ml [5μg/ml] for 2 h at 37°C). A PMP-resistant (PMP^r) variant was derived from the PMP^s parental strain by exposing the latter strain to PMP (10 μg/ml for 2 h at 37°C), followed by recovery and serial reexposure of surviving clones to PMP. In this manner, a variant that was resistant to PMP-induced killing (≥90% survival of a 2 × 10^3-CFU/ml inoculum following a 2-h exposure to PMP at 100 U/ml [5 μg/ml] at 37°C) was produced. The PMP^r phenotype was stable on multiple (n = 8) passages of the variant in PMP-free media.

TABLE 1. Phenotypic comparison of PMP^s and PMP^r *S. aureus* strains

Phenotypic trait	Value in:	
	PMP^s *S. aureus*	PMP^r *S. aureus*
Colonial morphology	Mucoid, convex; golden	Mucoid, convex; golden
PMP susceptibility (% survival[a])	≤10%	≥90%
Hemolysis (6.6% blood agar)	Weak β-hemolysis	Weak β-hemolysis
7.5% NaCl tolerance (MSA[b])	+	+
β-Lactamase	+	+
Catalase	+	+
Coagulase	+	+
Protein A	+	+
Clumping factor	+	+
AMP MIC	20 μg/ml	20 μg/ml
SUL MIC	160 μg/ml	160 μg/ml
AMP+SUL MIC (2:1 ratio)	2.5 μg/ml	1.25 μg/ml
Oxacillin MIC	16 μg/ml	16 μg/ml
Vancomycin MIC	0.125 μg/ml	0.125 μg/ml
Novobiocin (0.10 μg)	S[c]	S
Bacitracin (0.5 μg)	S	S
Mannitol Fermentation	+	+
Sucrose Fermentation	+	+
Raffinose Fermentation	−	−
Dextrose Fermentation	+	+

[a] Survival of a 10^3-CFU/ml inoculum following exposure to PMP (100 U/ml) at 37°C for 2 h.
[b] MSA, mannitol-salt agar.
[c] S. susceptible.

The PMP^s parent and PMP^r variant *S. aureus* strains were compared for identity by traditional phenotypic identification methods (Table 1), as well as by both pulsed-field gel electrophoresis of genomic DNA and immunoblotting of cell surface epitopes. Pulsed-field gel electrophoresis of PMP^s and PMP^r *Sma*I macrorestriction endonuclease chromosomal fragments was performed by David Persing, Mayo Clinic, Rochester, Minn., by using standard techniques (23) involving a CHEF-DR pulsed-field electrophoresis apparatus (Bio-Rad, San Francisco, Calif.). Immunoblot analysis of cell wall proteins of PMP^s and PMP^r strains was performed by Maury Mulligan, Long Beach Veterans Administration Hospital, Long Beach, Calif.) by a previously published technique (19). For these analyses, sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of staphylococcal cell wall protein preparations were transferred to a nitrocellulose membrane and subsequently probed with a pooled human polyclonal anti-staphylococcal typing antiserum. These pulsed-field gel electrophoresis and immunoblotting analyses revealed no detectable significant genomic or cell wall protein differences between the PMP^s parental and derived PMP^r variant strains (Fig. 1).

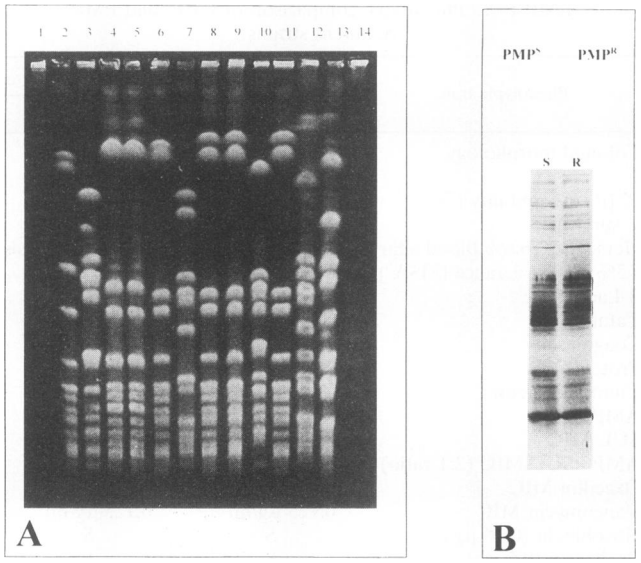

FIG. 1. Comparison of *S. aureus* SA-19 PMP^s (parental) and PMP^r (derived variant) strains by pulsed-field gel electrophoresis of *Sma*I macrorestriction genomic digests (A) and by pooled human polyclonal α-staphylococcal immunoblotting methods (B). (A) Lanes 4 and 5 demonstrate genomic identity between the PMP^s (lane 4) and PMP^r (lane 5) *S. aureus* strains, while lanes 2 and 3 and lanes 6 to 13 represent pulsed-field gel electrophoresis analysis of representative unrelated *S. aureus* strains (lanes 1 and 14 contain *Eco*RI-digested lambda phage molecular weight ladders). (B) Equivalence of the PMP^s and PMP^r *S. aureus* strain pair regarding surface protein composition.

Antibiotics and MICs. AMP and SUL were obtained in powder form from Pfizer, Inc., New York, N.Y., and suspended in appropriate buffer as directed by the manufacturer. Antibiotics were freshly suspended and filter sterilized (pore size, 0.22 μm) prior to use. The MICs of AMP and SUL (alone or in combination) in Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich.) supplemented with 2% (wt/vol) NaCl were determined by using a standard microtiter assay described previously (1, 15), with a final bacterial inoculum of 5×10^5 CFU/ml and a range of antibiotic concentrations of 0.625 to 1,280 μg/ml for AMP and SUL singly and 0.625:0.312 to 1,280:640 μg/ml, for AMP-SUL, respectively, in a 2:1 combination. MICs were defined as the lowest antibiotic concentrations inhibiting visible growth (1, 15). Under these conditions, MICs of AMP and SUL alone for *S. aureus* PMP^s and PMP^r strains were both 20 and 160 μg/ml, respectively, and MICs of the 2:1 combination of AMP+SUL were 2.5 and 1.25 μg/ml, respectively.

Fluorescence labeling for FACS analysis. (i) *S. aureus*. PMP^s and PMP^r *S. aureus* strains were cultured to late-logarithmic growth phase on a rotating drum for 12 to 14 h in MHB. Bacterial cells were harvested by centrifugation (3,000 × *g* for 10 min) and washed three times at 4°C in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02 M EDTA (TNE [pH 7.25]). Fluorescence labeling with Hoechst 33342 fluorochromatic dye (Polysciences, Warrington, Pa.), a DNA-intercalating dye, was performed as previously described (25, 33) and was followed by washing and resuspension in Tyrode's solution (pH 7.4) to a final concentration of 5×10^9 CFU/ml as determined by counting in a hemocytometer. Before being tested by flow cytometry, labeled bacterial suspensions were sonicated on ice at 60 Hz (sonifier model 350; Branson, Danbury, Conn.) for 4 s to ensure singlet organisms, washed twice in TNE, and sonicated again. Such sonication strategies were confirmed to yield essentially homogeneous singlet particle suspensions by light microscopy, and by flow cytometry as measured by forward light scatter (data not shown). Labeled, washed, and sonicated bacterial cells were recounted in a hemocytometer and adjusted to the desired final concentration. Hemocytometer-determined cell concentrations were confirmed by quantitative culture on 6.6% blood agar (Clinical Standards Laboratories, Los Angeles, Calif.). Neither Hoechst 33342 labeling nor sonication significantly altered the viability of either the PMP^s or PMP^r *S. aureus* strains.

(ii) Platelets. Platelets obtained from healthy human donors or New Zealand White rabbits were fluorescence labeled for fluorescence-activated cell sorter (FACS) analysis as described previously (25, 33). In brief, whole blood was freshly collected in polypropylene containers with buffered citrate anticoagulant (anticoagulant-to-blood ratio, 1:5) (25, 33) containing 1 μg of prostaglandin E_1 per ml to prevent platelet activation (final concentration, 0.35 mM; Sigma). PRP was produced as above, and the platelets in PRP then were labeled for 2 h at 20°C with 2.5 μg of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody P2 per ml, directed against platelet surface glycoprotein IIb/IIIa (Amac, Westbrook, Maine) (25, 33). Following labeling, the PRP supernatant was centrifuged at 2,000 × *g* (10 min), and the resulting platelet pellet was washed twice in citrate buffer (25, 33) to yield plasma-free, FITC-labeled platelets, which were counted by hemocytometry and resuspended to a final concentration of 4×10^8/ml in Tyrode's solution.

Analysis of *S. aureus* adherence to platelets. A modified cytofluorograph and cell sorter (FACS IV; Becton Dickinson, Mountain View, Calif.) were used to detect and quantify the binding of Hoechst 33342-labeled *S. aureus* cells to FITC-labeled platelets as previously described (33). Fluorescein (i.e., labeled platelets) was excited at 501 nm with an argon laser (Spectra Physics, Mountain View, Calif.) operating at 300 mW, and the resulting emission was collected through a 525 ± 25-nm band-pass filter. Hoechst 33342 (i.e., labeled *S. aureus* cells) was excited at 363 nm with a second argon laser, and the resulting emission was collected through a <460-nm short-pass filter. Unlabeled and labeled *S. aureus* cells alone or platelets alone were also analyzed in this system, and two-dimensional fluorescence profiles were generated as previously described (25, 33) by computer (DEC PDP11/73; Digital, Merrimack, N.H.) with specially designed software (Becton Dickinson).

In pilot studies, both human and rabbit platelets were tested for their interaction with *S. aureus*. No significant quantitative differences in *S. aureus* adherence to platelets from these two sources were observed. Therefore, to ensure donor reproducibility, rabbit platelets from the same donor animals were used in all subsequent flow cytometry studies. Binding studies were performed at 20°C by mixing Hoechst 33342-labeled *S. aureus* and FITC-labeled platelets in 1 ml of Tyrode's solution. At preselected times after mixing, binding was measured by flow cytometry and expressed as the percentage of *S. aureus* cells bound to platelets. Binding studies in which labeled PMP^s or PMP^r *S. aureus* strains were added to labeled platelets over a wide range of bacterium-to-platelet ratios (1:20 to 100:1) were performed to determine the ratio at which platelet binding by these organisms was maximal. These studies indicated that maximal adherence of each strain to platelets occurred at an *S. aureus*-to-platelet ratio of 10:1. This ratio was used in all subsequent investigations.

For all analyses, the percentage of *S. aureus* bound to platelets was calculated by dividing the number of dually

labeled particles (which represent *S. aureus* bound to platelets) by the total number of Hoechst 33342-labeled particles (i.e., bound and unbound *S. aureus*), and multiplying by 100. Data are expressed as mean values ± standard error and represent multiple experiments performed on different days.

Exposure of *S. aureus* to PMP prior to FACS. The effect of PMP on *S. aureus* adherence to platelets was also investigated by exposing 5×10^8 CFU labeled *S. aureus* cells per ml to PMP at sublethal concentrations (1.25 to 2.5 μg/ml) in Eagle's minimal essential medium for either 30 min or 2 h at 37°C. After PMP exposure, organisms were washed twice in Tyrode's solution, sonicated, adjusted to a final bacterium-to-platelet ratio of 10:1, and evaluated for their binding to platelets by FACS as described above. *S. aureus* cells exposed to PMP preparations inactivated by anionic affinity membrane adsorption (27, 33) were used as negative controls in these binding studies. Exposure of *S. aureus* strains to 1.25 or 2.5 μg of PMP per ml for 30 min at 37°C did not significantly alter viability as determined by quantitive culture, confirming that PMP concentrations and exposure times were sublethal (data not shown).

Exposure of *S. aureus* to antibiotics prior to FACS. A variety of investigations have shown that cell wall-antagonistic antibiotics (e.g., β-lactams, vancomycin) may reduce bacterial adherence to platelet-fibrin matrices in vitro and in the experimental infective endocarditis model (2, 12, 14, 16). Recent studies have shown that AMP (in contrast to oxacillin) has a relatively high binding affinity for the dominant penicillin-binding protein of oxacillin-resistant *S. aureus* (PBP-2a) (4) and that the combination of AMP with the β-lactamase inhibitor SUL is equivalent or superior to vancomycin in the prophylaxis and therapy of experimental oxacillin-resistant *S. aureus* infective endocarditis (15, 20). Thus, the current study employed PMP alone or in combination with AMP and/or SUL to investigate the activity of these agents on staphylococcal adherence to platelets in vitro.

To determine the effect of antibiotics on *S. aureus* adherence to platelets, PMP^s or PMP^r *S. aureus* cells (5×10^8 CFU/ml) were exposed to AMP (10 μg/ml), SUL (20 μg/ml), or a combination of AMP plus SUL (10 and 20 μg/ml, respectively) for 2 h at 37°C. These antibiotic concentrations represent readily achievable peak levels in rabbit serum, obtained during prophylaxis of experimental infective endocarditis (1). After antibiotic exposure, bacteria were washed twice in Tyrode's solution, sonicated, and processed as above for flow cytometry at bacterium-to-platelet ratios of 10:1. FITC-labeled *S. aureus* cells identically exposed to MHB lacking antibiotics were used as controls for respective flow-cytometric analyses.

Exposure of *S. aureus* to PMP and antibiotics prior to FACS. To investigate the combined effects of antibiotics and PMP on *S. aureus* adherence to platelets, we used two distinct strategies. In the first strategy, we examined the effect of concomitant PMP-plus-antibiotic exposures on *S. aureus* adherence to platelets. PMP^s or PMP^r cells (5×10^8 CFU/ml) were exposed to antibiotics as above (2 h at 37°C) and PMP (1.25 or 2.5 μg/ml, respectively), with the exposure of organisms to PMP concurrent with the last 30 min of antibiotic exposure. After this combined PMP-antibiotic exposure, bacterial cells were washed and prepared for analysis of platelet adherence by flow cytometry as described above. In the second strategy, PMP^s or PMP^r *S. aureus* cells (5×10^8 CFU/ml) were sequentially exposed to sublethal PMP concentrations in Eagle's minimal essential medium (2.5 μg/ml) for 30 min at 37°C as above, washed twice in Tyrode's solution, and exposed to AMP alone (10 μg/ml), SUL alone (20 μg/ml), or a combination of AMP plus SUL (10 and 20 μg/ml, respectively) in MHB for 2 h at 37°C. Following such exposure, organisms were washed, resuspended in Eagle's minimal essential medium, and sonicated as described above. After sequential exposure to PMP and antibiotics in this manner, *S. aureus* cells were prepared for flow-cytometric analysis of platelet binding as before.

Statistical analyses. Differences in *S. aureus* adherence to platelets were compared by using unpaired Student's *t* test, with Bonferroni correction for multiple comparisons. In each case, $P \leq 0.05$ was considered statistically significant.

RESULTS

Adherence of PMP^s and PMP^r *S. aureus* cells to platelets. Flow cytometry of Hoechst 33342-labeled PMP^s or PMP^r *S. aureus* cells alone, or FITC-labeled platelets alone, demonstrated that each cell type was well labeled and appeared as a single population (Fig. 2A and B). No overlap was detected in the emission spectra of labeled staphylococcal cells and labeled platelets, and forward light-scattering analyses confirmed that both *S. aureus* cells and platelets were predominantly singlet particles (data not shown). For both the PMP^s and PMP^r strains, flow-cytometric analysis of *S. aureus*-platelet mixtures revealed the emergence of a new and distinct population of dually labeled particles within 15 to 20 s of mixing, indicating that rapid binding between *S. aureus* cells and platelets had occurred (Fig. 2C). Moreover, the percentage of both the PMP^s and PMP^r *S. aureus* cells that bound to platelets within 1 min of mixing (e.g., mean 50.8 ± 5.1% adherence [$n = 3$]) was not significantly different from that observed after 30 min (e.g., mean 53.6 ± 4.8% adherence [$n = 2$]), indicating that maximal binding had occurred rapidly.

Effect of PMP on *S. aureus* adherence to platelets. To investigate the ability of PMP to influence *S. aureus* adherence to platelets, we exposed PMP^s or PMP^r *S. aureus* cells to sublethal concentrations of PMP (1.25 or 2.5 μg/ml) for 30 min and used flow cytometry to quantify their ability to subsequently bind platelets. Neither PMP^s nor PMP^r *S. aureus* cells exposed to PMP preparations rendered inactive (iPMP) via anionic membrane adsorption exhibited significant alteration in platelet adherence. In contrast, the ability of both *S. aureus* strains to adhere to platelets was markedly diminished following exposure to sublethal concentrations of bioactive PMP for 30 min, irrespective of the susceptibility of the strains to PMP-induced killing (Tables 2 and 3). For example, following exposure to PMP concentrations of 1.25 or 2.5 μg/ml, the percent adherence of the PMP^s strain to platelets was significantly reduced from 55.6% ± 3.2% to 12.2% ± 3.2% or 11.1% ± 2.3%, respectively (Table 2). Similarly, the percent adherence of the PMP^r *S. aureus* strain to platelets was also significantly reduced by exposure to PMP at either concentration (Table 2).

Effect of antibiotics on *S. aureus* adherence to platelets. To examine the effects of antibiotics on the ability of PMP^s and PMP^r *S. aureus* strains to adhere to platelets, were separately exposed organisms to AMP (10 μg/ml), SUL (20 μg/ml), or a combination of AMP plus SUL (10 and 20 μg/ml, respectively) for 2 h prior to measuring platelet adherence by flow cytometry. Compared with corresponding PMP^s control *S. aureus* cells exposed to antibiotic-free MHB, antibiotic-exposed cells exhibited no significant changes in percent platelet adherence (Table 2). Similarly, for the PMP^r strain, each of these antibiotic exposure regimens produced no significant changes in adherence of *S. aureus* cells to platelets. For both the PMP^s and PMP^r strains, there was a trend for increased platelet adherence following exposure to the AMP-SUL combination

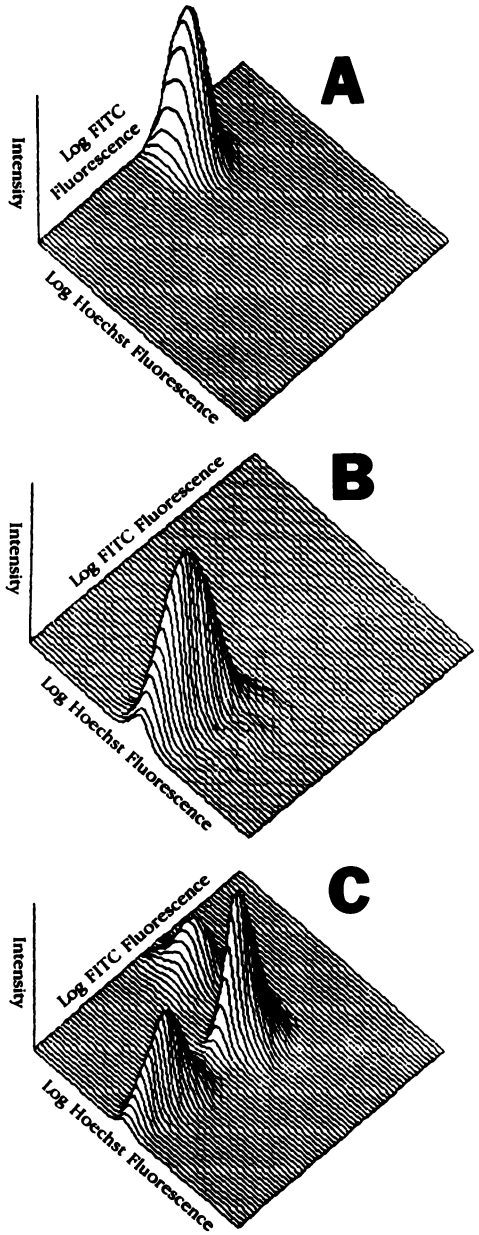

FIG. 2. Two-color flow-cytometric analysis of *S. aureus* adherence to platelets. (A) FITC-labeled platelets appear as a focused population of cells. (B) *S. aureus* cells labeled with Hoechst 33342 dye appear as a distinct population. (C) Addition of labeled *S. aureus* cells to labeled platelets (10:1) resulted in the emergence of a new population of dually labeled particles, indicating that adherence had occurred. In this representative FACS analysis, 63.8% of the platelets and 54.7% of the *S. aureus* cells were involved in binding.

(18.5 and 15.8% increases, respectively) although this did not reach statistical significance (Table 2).

Effect of PMP in combination with antibiotics on *S. aureus* adherence to platelets. To examine the net effects of PMP and antibiotics on *S. aureus* adherence to platelets, PMP^s or PMP^r strains were either concomitantly or sequentially exposed to antibiotics and PMP prior to quantitative analysis of platelet adherence by flow cytometry.

(i) Concomitant exposures. When the PMP^s *S. aureus* strain was exposed to PMP concomitantly with antibiotics, significant reductions in percent bacterium-platelet adherences were observed for each regimen as compared with iPMP-antibiotic exposures (Fig. 3; Table 3). For example, when the PMP^s strain was exposed to sublethal PMP concentrations (2.5 μg/ml) in the presence of antibiotics, reductions in platelet adherence from 47.9% ± 3.4% to 14.6% ± 1.6%, from 52.4% ± 4.0% to 9.0% ± 1.3%, and from 63.5% ± 7.6% to 12.2% ± 3.6% were observed for AMP, SUL, and AMP-SUL, respectively, compared with iPMP-antibiotic exposures ($P \leq 0.05$ for each condition [Table 3]). These reductions in ability to adhere to platelets were not significantly different from those produced by PMP alone (Tables 2 and 3).

Similarly, concomitant PMP-antibiotic exposure rendered the PMP^r *S. aureus* strain significantly less able to adhere to platelets for each antibiotic regimen, compared with cells exposed to iPMP plus antibiotics (Table 3). The magnitudes of reduction in platelet adherence for PMP^s and PMP^r cells were virtually equivalent (Table 3). Moreover, for both the PMP^s and PMP^r strains, addition of PMP during the last 30 min of AMP-SUL exposure reversed the increased adherence seen on AMP-SUL exposure alone (Tables 2 and 3; Fig. 3).

(ii) Sequential exposures. PMP is known to exert prolonged postexposure growth-inhibitory effects against *S. aureus* (29). We investigated whether such postexposure effects would also include reductions in bacterial adherence to platelets in the face of subsequent antibiotic exposures. In these studies, PMP^s and PMP^r strains were exposed sequentially to PMP and to antibiotics. For the PMP^s *S. aureus* strain, PMP exposure (2.5 μg/ml) followed by exposure to AMP, SUL, or AMP-SUL resulted in significantly reduced platelet adherence compared with controls. For example, compared with control PMP^s organisms exposed to iPMP alone followed by antibiotic-free MHB (72.7% ± 4.0% adherence to platelets), PMP exposure followed by antibiotic exposure significantly reduced bacterium-platelet adherence to 17.9% ± 3.1%, 37.4% ± 3.3%, or 50.6% ± 7.6% for PMP exposure followed by AMP alone, SUL alone, or AMP-SUL, respectively ($P \leq 0.05$ for each condition versus controls). Similar significant reductions in platelet adherence were observed for the PMP^r strain following sequential PMP-antibiotic exposures ($P \leq 0.05$ in all cases) (data not shown). Of note, exposure of both the PMP^s and PMP^r strains to PMP prior to AMP-SUL prevented the increased platelet adherence observed with AMP-SUL exposure alone (Table 2; Fig. 3). For SUL or AMP-SUL, reductions in percent platelet adherence of PMP^s and PMP^r cells concomitantly exposed to PMP plus antibiotics were significantly greater than those of identical cells exposed sequentially to PMP followed by antibiotics ($P = 0.02$ or 0.004, and 0.03 or 0.005 for PMP^s and PMP^r versus SUL or AMP-SUL, respectively).

DISCUSSION

PMP secretion is induced from platelets by physiological levels of thrombin in vitro (30); moreover, thrombin can be generated in vivo by release of local procoagulant molecules, such as tissue factor, from damaged endothelium (5, 6). In a recent series of investigations, we have demonstrated that PMP exerts a variety of antimicrobial effects upon common endovascular pathogens, such as *S. aureus* and *C. albicans*, including direct microbicidal effects, prolongation of postexposure staphylococcal growth inhibition, and synergistic enhancement of antibiotic-induced staphylococcal killing (27–30). PMP alone has also been shown to render *S. aureus* and *C. albicans* cells less adherent to platelets in vitro (32, 33). The importance

TABLE 2. Effect of PMP alone or antibiotics alone on adherence of *S. aureus* PMP^s and PMP^r strains to platelets[a]

Exposure	% Adherence[b]		% Change vs control		*P* vs control[c]	
	PMP^s	PMP^r	PMP^s	PMP^r	PMP^s	PMP^r
30 min iPMP (control)	55.6 ± 3.2	50.8 ± 4.1				
30 min PMP (1.25 μg/ml)	12.2 ± 3.2	15.2 ± 3.7	−78.1	−70.1	0.003	0.005
30 min PMP (2.5 μg/ml)	11.1 ± 2.3	10.6 ± 2.3	−80.3	−75.8	0.004	0.002
2 h MHB (control)	73.6 ± 6.5	74.5 ± 4.1				
2 h AMP	57.5 ± 6.4	58.8 ± 4.4	−21.9	−21.1	NS[d]	NS
2 h SUL	58.1 ± 4.2	57.1 ± 3.2	−21.1	−22.5	NS	NS
2 h AMPSUL[e]	87.2 ± 5.6	85.3 ± 6.6	+18.5	+15.8	NS	NS

[a] As determined by quantitative FACS analyses at an *S. aureus*-to-platelet ratio of 10:1 (see text).
[b] Mean percent adherence ± standard error (minimum $n = 2$).
[c] compares presence versus absence of bioactive PMP.
[d] NS, not significantly different from control ($P \geq 0.05$).
[e] AMP (10 μg/ml) in combination with SUL (20 μg/ml).

of this constellation of PMP effects has recently been underscored by Sullam et al. (24), who demonstrated that animals rendered selectively thrombocytopenic were less able to clear PMP-susceptible viridans streptococci from infected vegetations than were their counterparts with normal platelet counts.

Since adherence of *S. aureus* to platelets and vascular endothelium is considered pivotal in the pathogenesis of endovascular infection (e.g., infective endocarditis [7–10, 21]), the antiadherence properties of antibiotics and PMP may be critical in the overall in vivo efficacy of these agents. Therefore, the current study examined the modulation of *S. aureus* adherence to platelets when bacterial cells were exposed alone, sequentially or concomitantly to PMP and antistaphylococcal antibiotics.

The present study confirms that PMP reduces staphylococcal binding to platelets in the absence of plasma factors, such as fibrinogen, in vitro. Of importance, the antiadherence properties of PMP were shown to be distinct from its microbicidal effects, since PMP^s and PMP^r organisms were equally susceptible to PMP-induced inhibition of platelet adherence. In addition, antibiotic exposures of both the PMP^s and PMP^r strains produced, at most, modest increases in percent adherence to platelets, an observation similar to the results of our study in which oxacillin exposure resulted in increased platelet adherence of a different *S. aureus* strain (33). Moreover, the current data demonstrate that PMP antiadherence mechanism(s) are not antagonized by antibiotics; rather, PMP reverses antibiotic-induced increases in bacterial adherence to platelets. The specific mechanism(s) by which PMP inhibits, and antibiotics enhance, *S. aureus* adherence to platelets is not known but is currently under investigation in our laboratory, as are the potential activities of these agents in modulating staphylococcal adherence to platelets in the presence of plasma factors.

The synergistic bactericidal and bacteriostatic properties of PMP and antibiotics (27–30), as well as the antiadherence properties of PMP demonstrated in the present study, suggest that PMP may have an important in vivo role in defense against infective endocarditis, particularly in the setting of antibiotic prophylaxis. The mechanism(s) of antibiotic prophylaxis in infective endocarditis is neutrophil independent (3) and correlate with prolonged, antibiotic-induced growth-inhibitory properties of serum (1–3). Therefore, in light of the potent and diverse antimicrobial effects of PMP in the presence and absence of antibiotics, our collective data suggest that the local secretion of this peptide from platelets bound to damaged endothelium may well represent one important mechanism which is operative in antibiotic-mediated prophylaxis of endocarditis. This potential in vivo role of platelets and PMP in successful antibiotic prophylaxis of endocarditis has recently been supported by the studies of Fluckiger et al. in the experimental animal model (11).

TABLE 3. Effect of concomitant PMP and antibiotic exposure on *S. aureus* PMP^s and PMP^r strain adherence to platelets[a]

Exposure	% Adherence[b]		% Change vs control[c]		*P* vs control[d]	
	PMP^s	PMP^r	PMP^s	PMP^r	PMP^s	PMP^r
2 hMHB + 30 min iPMP	52.9 ± 8.2	53.8 ± 4.0				
2 h AMP + 30 min iPMP	47.9 ± 3.4	48.9 ± 3.7	−9.3	−9.2	NS[e]	NS
2 h AMP + 30 min PMP	14.6 ± 1.6	11.3 ± 2.3	−69.6	−76.9	0.001	0.007
2 h SUL + 30 min iPMP	52.4 ± 4.0	47.5 ± 3.5	−0.8	−11.2	NS	NS
2 h SUL + 30 min PMP	9.0 ± 1.3	10.2 ± 2.0	−82.9	−78.6	0.001	0.002
2 h AMP+SUL[f] + 30 min iPMP	63.5 ± 7.6	58.3 ± 4.6	+20.0	+8.3	NS	NS
2 h AMP+SUL + 30 min PMP	12.2 ± 3.6	11.7 ± 2.1	−81.0	−78.3	0.003	0.006

[a] As determined by quantitative FACS analyses at an *S. aureus*-to-platelet ratio of 10:1 (see text) and a PMP concentration of 2.5 μg/ml.
[b] Mean percent adherence ± standard error (minimum $n = 2$).
[c] Percent change in adherence versus control (iPMP exposure).
[d] compares presence and absence of bioactive PMP.
[e] NS, not significantly different from control ($P \geq 0.05$)
[f] AMP (10 μg/ml) in combination with SUL (20 μg/ml).

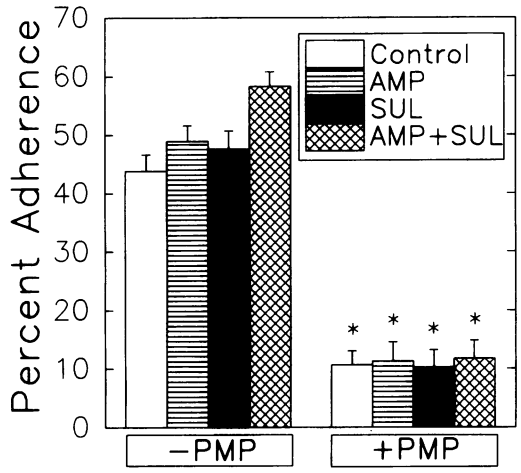

FIG. 3. Summary of the effects of PMP with or without antibiotics on adherence of PMP^r *S. aureus* cells to platelets in vitro. Data shown represent mean values of a minimum of three independent assays performed on different days. *, statistically significant reduction ($P \leq 0.05$); see the text for PMP and antibiotic exposure regimens.

ACKNOWLEDGMENTS

We thank Wendy Foss for her excellent technical contributions.

This study was supported in part by Pfizer Research Foundation Grant 92-S-0534 to A.S.B., a VA Career Development Program grant and National Institutes of Health grant AI-32506 to P.M.S., and American Heart Association Advanced Research Award 979-F1-2 and grant 1038.GI.1 to M.R.Y.

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