Staphylococcus aureus Susceptibility to Thrombin-Induced Platelet Microbicidal Protein Is Independent of Platelet Adherence and Aggregation In Vitro

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Bacterium-platelet interactions at the cardiac valve surface represent an important initial step in the induction of infective endocarditis (IE). This cell-cell interaction may play either a protagonistic role in the induction of IE via bacterial adherence to and aggregation of platelets or an antagonistic role via secretion of platelet-derived microbicidal molecules. We examined the spectrum and interrelationship of three aspects of the interaction of 20 clinical *Staphylococcus aureus* isolates with rabbit platelets in vitro: (i) *S. aureus* adherence to platelets; (ii) *S. aureus*-induced platelet aggregation; and (iii) *S. aureus* resistance to the action of thrombin-induced platelet microbicidal protein (PMP; low-molecular-weight cationic peptides contained in alpha granules). Among the 20 *S. aureus* isolates (11 bacteremia, 9 endocarditis), there was a heterogeneous distribution profile for each of the bacterium-platelet interaction parameters studied. For *S. aureus*-platelet adherence and *S. aureus*-induced platelet aggregation, 3 of 20 and 7 of 20 isolates tested were considered highly active for each respective parameter; 5 of 20 staphylococcal strains were deemed resistant to the bactericidal action of PMP. In addition, more endocarditis isolates (45%) were PMP resistant than strains from patients without endocarditis (19%). When analyzed concomitantly, there was a significant, positive correlation between *S. aureus*-platelet adherence and *S. aureus*-induced platelet aggregation among isolates ($P = 0.003$; $r = 0.78$). In contrast, there were no statistically significant relationships between either platelet adherence or aggregation and PMP resistance among these 20 *S. aureus* isolates. These data suggest that platelet adherence and aggregation are related abilities of *S. aureus*, while resistance to thrombin-induced PMP is an independent phenotypic characteristic and potential virulence factor.

Adherence of bacteria to target tissue surfaces is generally felt to represent a critical first step in the induction of infection (13–15). This paradigm has been extended to the induction of infective endocarditis (IE), in which circulating intravascular microorganisms must adhere to cardiac valve endothelium to initiate the process. Platelet-bacterium interactions at the cardiac valve surface are felt to be crucial in this regard, with the ability of bacteria to both adhere to and aggregate platelets in vitro correlating with induction of endocarditis in vivo (20, 21). However, recent data suggest that bacterium-platelet interactions also represent a mechanism of host defense by which cardiac valve-adherent organisms may be eradicated via the local elaboration of thrombin-induced platelet microbicidal protein (PMP) (9). In the present study, experiments were designed to quantitatively examine the spectra and interrelationships of three aspects of the interaction of *Staphylococcus aureus*, the most common cause of intravascular infections, with platelets in vitro: (i) *S. aureus* adherence to platelets; (ii) *S. aureus*-induced platelet aggregation; and (iii) the resistance of *S. aureus* to the antimicrobial action of thrombin-induced PMP.

MATERIALS AND METHODS

Bacterial isolates. Twenty *S. aureus* isolates were obtained from the Clinical Microbiology Laboratory, Harbor-UCLA Medical Center, from bacteremic patients with documented

staphylococcal infections. These 20 isolates were recovered from blood cultures from patients without evidence of endocarditis ($n = 11$) or with documented staphylococcal endocarditis ($n = 9$); the clinical classifications of endocarditis versus nonendocarditis bacteremias were defined as previously described (1). To account for the influence of *S. aureus* capsular biochemistry on interaction with platelets, capsular type was determined (as previously described [24]) for each of the isolates studied (courtesy of W. Karakawa, Pennsylvania State University, University Park). The sources and capsular types of these 20 *S. aureus* isolates are summarized in Table 1. There was only one isolate recovered from each patient; no isolate represented a common-source outbreak strain.

S. aureus isolates were grown on 6.6% sheep blood agar for 14 h, harvested, washed twice in phosphate-buffered saline (PBS, pH 7.2), centrifuged ($2,000 \times g$, 15 min), and resuspended in PBS. To disperse organism aggregates, bacterial suspensions were sonicated (~ 15 s at 60 Hz; model 350 Sonifier; Branson Sonic Power Co., Danbury, Conn.) and adjusted to concentrations appropriate for each assay (see below); bacterial concentrations within each suspension were determined spectrophotometrically. Quantitative cultures of bacterial suspensions before and after sonication were compared to ensure that sonication did not reduce organism viability.

***S. aureus*-platelet adherence.** (i) **Preparation of platelet ghosts.** Blood from the central ear artery of dedicated, healthy New Zealand White rabbits was freshly collected

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TABLE 1. Source and capsular phenotypes^a of *S. aureus* isolates studied

<i>S. aureus</i> isolate	Source	Capsule type
SA 1	Bacteremia ^b	8
SA 2	Bacteremia	8
SA 3	Endocarditis ^c	5
SA 4	Endocarditis	8
SA 5	Endocarditis	5
SA 6	Endocarditis	5
SA 7	Bacteremia	8
SA 8	Bacteremia	8
SA 9	Endocarditis	5
SA 10	Endocarditis	8
SA 11	Bacteremia	5
SA 12	Bacteremia	8
SA 13	Bacteremia	5
SA 14	Endocarditis	5
SA 15	Endocarditis	5
SA 16	Endocarditis	8
SA 17	Bacteremia	8
SA 18	Bacteremia	5
SA 19	Bacteremia	5
SA 20	Bacteremia	5

^a Determined as previously described (24).^b Isolated from blood cultures from bacteremic patients without endocarditis.^c Isolated from blood cultures from patients with documented *S. aureus* endocarditis (1).

into siliconized tubes containing citrate anticoagulant. Low-speed centrifugation ($75 \times g$) of blood samples produced a loose erythrocyte pellet and an upper, platelet-rich plasma (PRP) supernatant. Collection of the upper two-thirds of the PRP supernatant routinely yielded platelets having <1% leukocyte contamination. To determine the platelet adherence capacity of *S. aureus* isolates, we prepared nonaggregating platelet ghosts as previously described (20, 21). Briefly, platelets in PRP were allowed to outdate for a minimum of 5 days to a maximum of 14 days (4°C), pelleted by centrifugation for 10 min at $2,000 \times g$ (4°C), and washed twice in 0.02 M Tris-HCl (pH 7.25) with 1.0% EDTA. The resulting platelet ghost pellet was washed twice in PBS and resuspended. Washed platelet ghosts were then resuspended to an optical density at 620 nm (OD_{620}) of 0.85 in PBS (Spectronic 401 spectrophotometer; Milton Roy Analytical Division, Rochester, N.Y.), which corresponded to $\sim 5 \times 10^8$ platelet ghosts per ml as measured by a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), and stored at 4°C . Responsiveness of the senescent platelet ghosts was verified by calcium stimulation, which reconstituted near-normal ADP-induced aggregation in platelet-free plasma. In pilot studies, bacterium-platelet adherence profiles were identical regardless of platelet outdating time (5 days versus 14 days).

(ii) ***S. aureus*-platelet adherence assay.** *S. aureus*-platelet adherence was determined by a modification of the spectrophotometric assay of Herzberg et al. (20, 21). Equal volumes (100 μl) containing concentrations of *S. aureus* and platelet ghosts appropriate to achieve *S. aureus*/platelet ghost ratios of 1:1, 10:1, and 50:1 were mixed in V-well microtiter assay plates and incubated for 30 min at 4°C . The plates were then centrifuged at $55 \times g$ for 5 min at 4°C to pellet only bacterium-platelet complexes. Control wells contained 200 μl of either platelet ghosts alone or washed bacteria alone. After centrifugation, 100- μl supernatant aliquots were sampled from wells and diluted into 1.9 ml of PBS; the OD_{700} of each supernatant sample was determined spectrophotomet-

rically, and the percentage of *S. aureus*-platelet adherence was calculated by using the following formula (20):

$$1 - \frac{\text{OD}_{\text{reaction supernatant}}}{0.5 \times (\text{OD}_{\text{bacterial supernatant}} + \text{OD}_{\text{platelet supernatant}})} \times 100$$

For each *S. aureus* isolate, adherence assays were performed in triplicate on the same day, and the final percent adherence was calculated as the mean percent adherence $\pm$ standard deviation. On the basis of both pilot data from our own laboratory and previously published studies (21), *S. aureus* isolates exhibiting $\geq 15\%$ platelet adherence were considered highly active platelet adherers.

***S. aureus*-platelet aggregation assay.** A dual-channel platelet aggregometer (Peyton Associates, Buffalo, N.Y.) attached to a chart recorder was used to assess *S. aureus*-induced platelet aggregation by turbidimetry (7). For use in platelet aggregation assays, PRP was freshly obtained as described above; platelet concentrations in PRP were determined with a Coulter Counter and adjusted to $\sim 2.5 \times 10^8/\text{ml}$ ($\sim 250,000/\text{mm}^3$). PRP (250 μl) was warmed to 37°C in a glass microcuvette stirred at 900 rpm with a magnetic stirring microbar; all glass components were siliconized to prevent platelet contact activation. Platelet-poor plasma (PPP) obtained by centrifugation of PRP ($1,000 \times g$, 10 min) was used to establish the 100% light transmission baseline, while PRP served as the 0% aggregation baseline. A concentration-standardized volume of washed *S. aureus* suspension in PBS was then added to yield a final bacterium/platelet ratio of 10:1; this ratio provided optimal platelet aggregation in pilot studies in our laboratory. Light transmission was recorded for 25 min, or until platelet aggregation was complete as indicated by a minimum of 10 min of recorder chart equilibrium (no change in OD).

S. aureus-induced platelet aggregation was quantified via direct measurement of actual aggregation time (AAT) and change in OD (ΔOD) (Fig. 1). AAT was defined as the time from onset to completion of aggregation as defined above. Values for ΔOD were calculated as the maximum positive change in light transmission obtained from the chart recorder aggregation tracing. Control platelet aggregation profiles were produced for each experimental session by the addition of 25 μl of ADP reagent ($[\text{ADP}] = 2 \times 10^{-5} \text{ M}$; Sigma Chemical Co., St. Louis, Mo.) to 250- μl PRP samples, and these values were used in subsequent quantification of the aggregation parameters below. Additional control platelet aggregation profiles were produced for each experiment by using an *S. aureus* strain (Cowan 1; SA-1) with known platelet aggregation ability (complete aggregation within 5 min). Percent platelet aggregation, aggregation velocity, and aggregation index were respectively derived for each staphylococcal strain by using the following formulas:

$$\text{Percent aggregation} = \frac{\Delta\text{OD}_{\text{experimental}}}{\Delta\text{OD}_{\text{ADP control}}} \times 100$$

$$\text{Aggregation velocity} = \frac{\Delta\text{OD}_{\text{experimental}}}{\text{AAT}}$$

$$\text{Aggregation index} = \text{percent aggregation} \times \text{aggregation velocity}$$

ADP- and *S. aureus*-induced control platelet aggregation profiles were used to establish comparative maxima for each

FIG. 1. Parameters used to quantify *S. aureus*-induced platelet aggregation (AGG). AAT was measured as the time (minutes) elapsed between the beginning and completion of aggregation. AAT was derived from the distance measured on the recorder chart converted to real time by incorporating the chart speed. The overall change in OD (Δ OD) was measured as the difference between baseline OD and the OD of the sample at the point of recorder chart equilibrium after aggregation. Percent aggregation, aggregation velocity, and aggregation index were then computed by using AAT and Δ OD (see text).

parameter, normalized to 100% aggregation, 10 U/min (aggregation velocity), and 1,000 index units (aggregation index). Mean platelet aggregation values $\pm$ standard deviations were determined for each of the organisms studied from triplicate experiments performed independently on different days. On the basis of extensive pilot studies in our laboratory, a platelet aggregation index of 300 U was considered the breakpoint between good and poor platelet aggregators among staphylococcal strains.

Preparation of thrombin-induced PMP. PMP was prepared and assayed for bioactivity as previously described (9, 32). Briefly, rabbit PRP was obtained as described above and centrifuged for 10 min at $2,000 \times g$. The resulting platelet pellet was washed twice in Tyrode salts solution (0.08 mM NaCl, 3.8 mM K_2HPO_4 , 4.0 mM Na_2HPO_4 , 2.8 mM glucose, 16.6 mM citric acid, 34 mM sodium citrate, pH 6.8) (Sigma Chemical Co.) and resuspended in Eagle's minimal essential medium (MEM) to a concentration of $\sim 10^8$ platelets per ml as determined by a Coulter Counter and/or spectrophotometry ($\lambda = 600$ nm). Preparations rich in PMP were produced from washed platelets as previously described (9, 32) by thrombin stimulation of $\sim 10^8$ platelets per ml in MEM (1 U of thrombin [Sigma Chemical Co.] with 12.5 μ l of 0.2 M $CaCl_2$ per ml of washed platelet suspension; 20 min, 37°C) (9, 30, 32). Residual platelet material was removed by centrifugation ($2,000 \times g$, 10 min), and the PMP-rich supernatant was recovered (32).

Determination and standardization of PMP bactericidal activity. The total protein content of thrombin-induced PMP preparations was determined spectrophotometrically (25), and PMP bactericidal activity was assessed by using techniques modified from those of Donaldson and Tew (10). Briefly, PMP bioactivity assays were performed with *Bacillus subtilis* (ATCC 6633), an indicator organism highly sensitive to the bactericidal action of PMP (9, 32). For these studies, *B. subtilis* was cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C for 12 to 14 h; organisms were harvested by centrifugation, washed twice

in normal saline, and resuspended in PBS prior to use. All PMP bioactivity assays were performed in low-protein-binding microtiter plates (Corning Glass Works, Corning, N.Y.). A *B. subtilis* inoculum of 10^4 CFU/ml was added to microtiter wells containing a range of dilutions of the PMP-rich preparation to achieve a final inoculum of 10^3 CFU/ml and a final range of PMP dilutions from 1:1 to 1:1,024 (final well volume = 200 μ l). One well contained *B. subtilis* in MEM alone as a positive growth control. The microtiter plates were then incubated in ambient CO_2 (37°C). At 0, 5, 30, 60, and 120 min of incubation, 20- μ l aliquots were removed from each well, diluted into PBS containing 0.01% sodium polyanethanol sulfonate (an inhibitor of PMP-induced *B. subtilis* killing [32]), briefly sonicated (15 s, 60 Hz) to disperse organism agglutination, and quantitatively cultured onto 6.6% sheep blood agar. Kill curves were constructed comparing percent *B. subtilis* survival over time. Thrombin (1 U/ml) in PBS or MEM and supernatants from washed platelets not exposed to thrombin were used as additional controls in the PMP bioactivity assays. For these studies, PMP bioactivity was defined as the inverse of the highest PMP dilution (units per milliliter) which retained $\geq 95\%$ lethality versus *B. subtilis* (10). The PMP specific activity was then defined and quantified as PMP bioactivity units per milligram of protein.

PMP susceptibility of *S. aureus*. For use in PMP susceptibility assays, the *S. aureus* isolates ($n = 20$) were grown in brain heart infusion broth for 12 to 14 h at 37°C, harvested by centrifugation, washed twice in normal saline, and resuspended in PBS. Thrombin-induced PMP was then added to *S. aureus* suspensions (prepared as above) in low-protein-binding microtiter plates to achieve a final PMP concentration of 100 U/ml (specific activity, ~ 12.5 U/mg of protein) and a final bacterial inoculum of $\sim 10^3$, 10^5 , or 10^8 CFU/ml in parallel studies (final volume = 200 μ l). These differing *S. aureus* inocula were used to simulate intravascular bacterial densities observed in early, developing, and well-established endocarditis (13, 15). At 0, 30, 60, and 120 min of incubation at 37°C, 20- μ l aliquots were sampled from each microtiter well, diluted into PBS containing 0.01% sodium polyanethanol sulfonate (as above), and quantitatively cultured on 6.6% sheep blood agar; kill curves were then constructed comparing *S. aureus* survival over time. On the basis of extensive pilot data, we designated a breakpoint for *S. aureus* PMP susceptibility as $\leq 40\%$ survival of the initial inoculum (10^3 CFU/ml) after 2 h of exposure to the PMP preparation. Control *S. aureus* cultures were added to MEM without PMP and assayed in parallel.

Statistical analyses. Platelet adherence, platelet aggregation, and PMP susceptibility among the *S. aureus* isolates in this study were correlated by using Pearson's correlation, Spearman's rank correlation, and logistic regression analyses employing BDMP software accessed through a VAX mainframe. In addition, Student *t* tests were performed to compare individual isolate parameters. For each analysis, a *P* value of ≤ 0.05 was considered statistically significant; a correlation coefficient (*r*) of ≥ 0.5 was interpreted as significant.

RESULTS

***S. aureus*-platelet adherence.** Interaction with platelet ghosts allowed determination of *S. aureus* platelet adherence in the absence of aggregation. Variability in day-to-day adherence of platelets by respective *S. aureus* isolates was minimal ($\leq 5\%$), and there were no significant differences in

FIG. 2. Distribution of platelet adherence (PLT ADH) among the 20 *S. aureus* isolates studied. Highly active platelet adherers (●) were distinguished from less-active platelet adherers (○) by means of a 15% platelet adherence breakpoint.

bacterial adherence to platelets from the two dedicated platelet donor animals used. The 20 *S. aureus* human bacteremic isolates in this study exhibited heterogeneity in their ability to adhere to rabbit platelets at a bacterium/platelet ratio of 1:1 (Fig. 2). Two isolates lacked detectable platelet adherence, while three isolates were found to be capable of very high (~20%) platelet adherence. The remaining 15 isolates fell into a relatively normal distribution with regard to this platelet interaction parameter. Dose-response platelet adherence studies were also performed with three *S. aureus* isolates representing low and high platelet adherers (SA-4 and SA-18, respectively) and a well-characterized laboratory strain (Cowan, SA-1). Measurement of platelet adherence for these isolates at bacterium/platelet ratios of 1:1, 10:1, and 50:1 demonstrated that maximal adherence occurred at a ratio of 10:1 (Fig. 3). At a bacterium/platelet ratio of 50:1, the overall percentage of bacterial cells adhering to platelets decreased in comparison with adherence values observed at ratios of 1:1 and 10:1, suggesting that adherence of *S. aureus* to platelets was a saturable interaction. For example, for SA-1, percentages of bacterial adherence at 1:1 and 10:1

FIG. 3. Dose-response curves for *S. aureus* adherence to platelet ghosts. Regardless of the native platelet adherence of each isolate (positive or negative for adherence), emergence of peak adherence at a bacterium/platelet ratio of 10:1, beyond which adherence decreased, indicates saturation. Data shown represent mean percent adherence values $\pm$ standard deviations (bars) from four experiments.

FIG. 4. Distribution of platelet aggregation (PLT AGG) indices among the 20 distinct *S. aureus* isolates studied. A platelet aggregation index of 300 U was used as a breakpoint to distinguish those isolates exhibiting relatively high (●) versus relatively low (○) platelet aggregation indices.

were $23.5\% \pm 4.8\%$ and $33.2\% \pm 5.2\%$, respectively; in contrast, at a bacterium/platelet ratio of 50:1, the percent adherence substantially diminished to $9.1\% \pm 2.7\%$. There was no correlation between capsular type and platelet adherence among the 20 *S. aureus* isolates. In no case did sonication of *S. aureus* isolates alter subsequent platelet adherence.

***S. aureus*-induced platelet aggregation.** Platelets from the two dedicated donor animals were highly similar in response to ADP- and *S. aureus*-induced aggregation; minimal day-to-day variations ($\leq 5\%$) were independently controlled in each experimental session. For each aggregation parameter quantified, the *S. aureus*-induced platelet aggregation profiles were heterogeneously distributed. Compared with control ADP-induced platelet aggregation (normalized to 100%), percent *S. aureus*-induced platelet aggregation values ranged from a low of 22.0% to a high of 112.2%, with a mean percent aggregation value among the 20 isolates of 73.5%. Aggregation velocities also fell along a relatively normal distribution curve, with a low of 0.42 U/min and a high of 8.39 U/min; the mean aggregation velocity was 3.17 U/min. Aggregation indices, which incorporate both the degree (percent) and rate (velocity) of aggregation, yielded an overall assessment of *S. aureus*-induced platelet aggregation (Fig. 4). Aggregation index values ranged from a low of 18.5 to a high of 941.3 index units, with a mean of 260.9 units. In the majority of isolates, organisms capable of complete platelet aggregation ($\geq 100\%$ versus ADP-induced control) also exhibited relatively high aggregation velocities and aggregation indices; however, several isolates were divergent from this pattern. For example, one strain exhibited complete platelet aggregation (105.2%) but prolonged aggregation velocity (2.12 U/min), thereby yielding a low aggregation index of 223.0 index units. Overall, on the basis of the distribution of aggregation indices, 7 of the 20 *S. aureus* isolates studied were considered highly active platelet aggregators (aggregation indices of ≥ 300). There was no detectable correlation between capsular type and platelet aggregation among the *S. aureus* isolates studied, and there were no differences in the pre- and postsonication abilities of isolates to aggregate platelets.

Susceptibility of *S. aureus* to PMP. Each *S. aureus* isolate was independently assayed for PMP susceptibility in tripli-

FIG. 5. Distribution of *S. aureus* isolate susceptibility to PMP. Isolates were considered resistant to the bactericidal action of PMP if $\geq 40\%$ of the original inoculum (10^3 CFU/ml) survived 2 h of exposure to 100 U of PMP per ml at 37°C (●); isolates were otherwise considered PMP susceptible (○).

cate, and the mean survival percentages at 2 h were compared. At all inocula tested (10^3 , 10^5 , or 10^8 CFU/ml), the 20 *S. aureus* isolates were heterogeneous in PMP susceptibility (Fig. 5). At an inoculum of 10^3 CFU/ml, staphylococcal survival ranged from a low of 6.5% to a high of 90.6%. On the basis of the PMP susceptibility breakpoint of 40% survival of the original inoculum (10^3 CFU/ml) at 2 h, 75% of the *S. aureus* isolates tested were considered to be susceptible to the bactericidal action of PMP (PMP^s), while 25% were considered PMP resistant (PMP^r). Similar heterogeneity in PMP susceptibility was seen at the higher *S. aureus* inocula (10^5 and 10^8 CFU/ml), although the relative percent survival of each respective isolate increased with the higher initial inoculum size. *S. aureus* exposed to MEM alone or MEM containing 1 U of thrombin per ml exhibited no decrease in cell viability over the 2-h assay period. No correlation was detectable between capsular type and PMP susceptibility among the *S. aureus* isolates studied.

Correlation of *S. aureus* platelet adherence, aggregation, and PMP susceptibility. Multivariate logistic regression analyses, evaluating the interrelationships of the above three platelet interaction parameters, revealed a significant, positive correlation between *S. aureus* platelet adherence and platelet aggregation ($P = 0.003$, $r = 0.78$) (Fig. 6). In contrast, there were no correlations observed between platelet adherence and PMP susceptibility ($r = 0.28$) (data not shown) or platelet aggregation and PMP susceptibility ($r = 0.31$) (data not shown). Isolates recovered from patients with endocarditis were substantially more likely to be PMP resistant than isolates from nonendocarditis patients (45% versus 20% PMP resistant, respectively). Logistic regression analyses comparing PMP susceptibility with clinical syndromes (bacteremia versus endocarditis) also identified the trend of PMP resistance in association with endocarditis isolates; however, this trend did not achieve statistical significance ($P = 0.20$) given the sample size used in this study.

DISCUSSION

The ability of bacteria to interact with platelets is an important factor in the induction and development of intravascular infections, including IE (13, 22, 23). Bacterial pathogens may exploit platelets as a means of initial adher-

FIG. 6. Correlation of *S. aureus*-platelet adherence (PLT ADH) and *S. aureus*-induced platelet aggregation (PLT AGG). Individual isolate platelet adherence and platelet aggregation index values were compared by using Student's *t* test and subsequently ranked; Pearson's correlation and Spearman's rank correlation analyses were then used to determine correlation of these two parameters ($r = 0.78$, $P = 0.003$).

ence to damaged cardiac endothelium; moreover, the capacity of such pathogens to aggregate platelets and become embedded within platelet-fibrin vegetations likely contributes to evasion of phagocytic- and antibiotic-mediated bacterial clearances in IE. Conversely, platelets may actively participate in nonphagocytic antibacterial host defense in IE via secretion of alpha-granule-derived microbicidal substances such as PMP (9, 10, 30, 32). Therefore, bacteria which utilize platelet adherence and platelet aggregation during the initial induction stages of IE would potentially derive an additional survival advantage by being resistant to the bactericidal effects of PMP. In the present study, we evaluated the interrelationship among staphylococcal platelet adherence, platelet aggregation, and resistance to the bactericidal effects of thrombin-induced PMP.

Several interesting observations emanated from this investigation. Utilizing clinically significant bacteremic *S. aureus* isolates from patients with and without endocarditis, we observed a heterogeneity in the ability of these strains to both adhere to and aggregate platelets, as well as in their ability to resist the bactericidal action of PMP. Platelet adherence studies performed at various *S. aureus*/platelet ratios revealed this interaction to be saturable; we noted a similar saturability using quantitative flow cytometric analysis of *S. aureus*-platelet binding (31). This latter study also confirmed the adherence of *S. aureus* to platelets to be rapid and reversible, suggesting a receptor-ligand interaction. In the present study, only a minority of the *S. aureus* strains possessed these potentially important platelet interaction parameters; thus, only 15 and 35% of strains, respectively, were able to either significantly adhere to or aggregate platelets, while only 25% of the strains were resistant to the bactericidal action of PMP. This suggested that not all *S. aureus* strains are equally capable of producing IE in vivo, a conclusion supported by strain-to-strain variabilities in the 90% infective dose values for inducing experimental staphylococcal IE (17, 22). Also, when statistically analyzed in a pairwise fashion for each individual isolate, our data clearly show that the ability of a strain to adhere to platelets is significantly associated with its capacity to aggregate platelets.

In contrast, among individual staphylococcal strains, resistance to the bactericidal action of PMP was not statistically related to the ability of such strains to either adhere to or aggregate platelets. These data suggested that, for an individual *S. aureus* strain, the ability to adhere to and aggregate platelets is related in *S. aureus*, while resistance to the bactericidal action of PMP may be an independent phenotypic characteristic and possible virulence factor for these organisms. The observed trend that *S. aureus* isolates recovered from IE patients were more frequently PMP resistant suggests that in vivo PMP resistance in *S. aureus* provides those organisms a selective survival advantage in the pathogenesis of IE. The lack of statistical significance achieved in the relationship between *S. aureus* clinical syndrome (IE versus non-IE) and PMP susceptibility may be a function of the relatively small number of strains evaluated ($n = 20$); it also suggests that other host defense factors (e.g., polymorphonuclear leukocyte killing) contribute to modulation of the induction and progression of IE.

Although there is a considerable body of information concerning *S. aureus* adherence to a variety of biological surfaces, including endothelial cells (26) and matrix proteins such as fibrinogen and fibronectin (4, 18, 19, 23, 27, 31), little has been published on the mechanisms involved in staphylococcal adherence to and aggregation of platelets. For another IE pathogen, the viridans group streptococci, Sullam et al. (28, 29) have shown that platelet adherence occurs in the absence of plasma factors, is not inhibitable by monoclonal antibody blockade of the platelet Fc receptor, and is rapid, saturable, and reversible, suggesting a receptor-ligand interaction. In contrast, these same investigators have documented that viridans streptococcus-induced platelet aggregation depends on the presence of organism-specific immunoglobulin G in consort with other plasma factors (29). Herzberg et al. (20, 21), similar to our present findings, have shown that for viridans streptococci, the ability to adhere to platelets correlates with the capacity of such strains to aggregate platelets; they did, however, observe several streptococcal strains that adhered well to platelets without causing platelet aggregation. These latter data, as well as our own findings, suggest that bacterial platelet adherence and eventual bacterium-induced platelet aggregation are covirulence factors, but may not be mechanistically linked.

Clawson and colleagues (5–8) have examined the entire process of bacterium-induced platelet aggregation, from initial organism-platelet contact to irreversible aggregation. In these studies, they noted *S. aureus* to be the most potent bacterial platelet aggregator among the organisms studied and suggested that the initial preaggregation lag phase following bacterial exposure to PRP likely involved interaction of the organism with platelet surface receptors during an early contact phase (7). Subsequent bacterium-induced platelet aggregation then depended on adequate divalent cation concentrations, as well as platelet extracellular secretion of the nucleotide ADP. However, data from Sullam et al. (28) as well as from our own laboratory (31), utilizing fluorescence-activated cell sorting, confirm that the adherence of viridans streptococci and staphylococci to platelets is essentially complete very early in the contact phase and cannot solely account for the entire preaggregation lag period. The above studies suggest that bacterium-platelet adherence facilitates eventual platelet aggregation; however, the mechanism(s) by which the adherence and aggregation processes are linked remained poorly defined.

The elaboration of tissue factor with thrombinlike procoagulant activities at the surface of bacterium-colonized val-

vular endothelium is felt to be crucial in the growth and propagation of vegetations in IE (3, 11, 12). Of interest, recent work from our laboratory (31) as well as from Dankert (9) suggests that such thrombinlike substances are able to induce platelets to secrete low-molecular-weight cationic proteins which are bactericidal against both staphylococci (32) and the viridans streptococci (9). In vivo studies also implicated these thrombin-induced PMPs as important arbiters in rendering prophylactic efficacy in experimental viridans streptococcal IE (9). Similarly, recent studies from Berney and Francioli (2), using a neutropenic model of IE, have indirectly implicated platelet-derived factors in mediating effective antibiotic prophylaxis of experimental viridans streptococcal IE. There have been few previous data on the effect of these PMPs against *S. aureus*. Our present investigation shows that among clinically relevant *S. aureus* bloodstream isolates, there is a substantial heterogeneity in their susceptibility to the killing action of such a PMP(s) and that PMP resistance of individual staphylococcal strains is probably not linked to the capacity of such strains to either adhere to or aggregate platelets. Preliminary work in our own laboratory has shown that these PMPs are indeed cationic, of low molecular weight, heat stable, and distinct structurally and microbiologically from lysozyme (30, 32). This microbicidal protein, which we have termed PMP, has also been called thrombodefensin by Dankert (9); however, inclusion of these proteins into the defensin class of molecules requires them to possess specific structural features (16), and the use of the term thrombodefensin may be premature. A detailed structure-function analysis of these PMPs is presently under way in our laboratory. The interrelationships of platelet adherence, aggregation, and bacterial resistance to PMP in the induction and evolution of IE in vivo await further studies in relevant experimental animal models.

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