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Diminished Platelet Binding In Vitro by *Staphylococcus aureus* Is Associated with Reduced Virulence in a Rabbit Model of Infective Endocarditis

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The direct binding of platelets by bacteria is a postulated central mechanism in the pathogenesis of endocarditis. To address the role of binding more definitively, we employed Tn*551* insertional mutagenesis of *Staphylococcus aureus* parental strain ISP479 to generate an isogenic variant (strain PS12) that bound platelets minimally. As compared with the binding of ISP479, the binding of PS12 to platelet monolayers was reduced by 67.2%. Similarly, the binding of PS12 to platelets in suspension was reduced by 71.3%, as measured by flow cytometry. The low-binding phenotype was transducible into both ISP479 and *S. aureus* Newman. Southern blotting indicated that a single copy of Tn*551* was inserted within the chromosomes of PS12 and the transductants. When tested in a rabbit model, animals inoculated with PS12 were significantly less likely to develop endocarditis and had lower densities of organisms (CFU per gram) within vegetations and a decreased incidence of renal abscess formation, as compared with animals inoculated with the parental strain. The diminished virulence of PS12 was not attributable to a reduction in the initial attachment of organisms to the damaged endocardium, since 30 min after inoculation, PS12-infected animals had microbial densities on the valve surface comparable to those seen with the parental strain. These results indicate that the direct binding of *Staphylococcus aureus* to platelets is a major determinant of virulence in the pathogenesis of endocarditis. Staphylococcus-platelet binding appears to be critical for pathogenetic events occurring after the initial colonization of the valve surface, such as vegetation formation and septic embolization.

The direct binding of microorganisms to platelets is a postulated central mechanism in the pathogenesis of infective endocarditis (22). Platelet binding may be important for the initiation of endocardial infection, since platelets on the damaged valve surface may serve as binding foci for organisms circulating in the blood. A number of endocardial pathogens, including *Staphylococcus aureus*, *Streptococcus sanguis*, and *Candida albicans*, can bind platelets directly in vitro, suggesting that a similar interaction may occur in vivo (12, 24, 28, 29). Similarly, platelets augment the adherence of *S. aureus* to plastic intravenous catheters in vitro, also indicating that an analogous mechanism may enhance staphylococcal attachment to the endocardial surface (14). Moreover, in an animal model of endocarditis, rabbits given an inhibitor of platelet aggregation (aspirin) prior to inoculation with *S. aureus* had lower concentrations of bacteria (CFU per gram) within vegetations 24 h after infection, further suggesting that platelets facilitate microbial adhesion to the valve (18).

In addition to mediating the initial attachment of organisms to the endocardium, the adhesion of bacteria and platelets may also be important for the subsequent formation of mature vegetations. Since numerous organisms can bind platelets directly in vitro, it is possible that in vivo, such binding may result in the further accumulation of platelets onto the infected surface or in the continued deposition of bacteria via endocardial reseeding. Support for these mechanisms of vegetation formation comes predominantly from histologic studies of animals with experimental endocarditis, in which a progressive accumulation of platelets and bacteria at the outer surfaces of maturing lesions has been noted (10). Moreover, the induction of selective thrombocytopenia in rabbits with early streptococcal endocarditis results in vegetations of significantly smaller mass (23), further indicating that platelets are a major structural component of vegetations and that these cells continue to be deposited on the valve surface during the course of infection.

Although the studies described above have provided inferential evidence that microbe-platelet binding is important in the pathogenesis of infective endocarditis, more direct evidence that this interaction occurs in vivo has been lacking. Moreover, the molecular basis for microbial binding to platelets and its impact on disease progression have not been examined. For these reasons, we sought to address more definitively the mechanisms for the direct binding of *S. aureus* to human platelets and to determine the role of such binding in the pathogenesis of infective endocarditis.

MATERIALS AND METHODS

Bacterial strains, plasmids, DNA probes, and phages. The bacterial strains, phages, plasmids, and DNA probes used in this study are listed in Table 1.

Media and antibiotics. CYGP, 0.3 GL, and brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broths and agars were used as culture media (6). Erythromycin and ampicillin were used at concentrations of 15 μg/ml; sensitivity to cadmium nitrate was tested at a 0.1 mM concentration.

Assay for staphylococcal binding to platelet monolayers. Washed human platelets were prepared from fresh blood donated by healthy volunteers, as described previously (25). In brief, 5 volumes of blood were drawn into a syringe containing 1 volume of citrate anticoagulant and 1 μg of prostaglandin I_2 (Sigma Chemical Co., St. Louis, Mo.). Platelets were isolated by centrifugation (100 ×

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TABLE 1. Bacterial strains, plasmids, and DNA probes

Type	Comments
S. aureus strains	
ISP479	Parental strain carrying pI258
ISP479C	ISP479 cured of pI258
PS12	Tn*551*-derived, isogenic mutant of ISP479 with low-platelet-binding phenotype
Mutants A and B	Transductants of ISP479C with low-platelet-binding phenotype
Newman	Wild-type strain
Mutants I and J	Transductants of Newman with low-platelet-binding phenotype
Plasmids and probes	
pI258	Thermosensitive plasmid containing Tn*551*
pLTV1	Plasmid containing Tn*917*, with selectable markers for *E. coli* and *S. aureus*; a 1.5-kb *Hin*dIII gel-purified fragment or a 5.6-kb *Eco*RI fragment of pLTV1 was used as a probe for Tn*551*
agrA probe	513-bp fragment of *agrA* (nucleotides 3830–4342) (16)
sar probe	732-bp fragment of *sarA* (3)

g, 15 min), followed by washing, fixation in 0.8% formaldehyde (30 min at 37°C), additional washing, and suspension in Tyrode's solution. Platelets prepared in this manner appeared morphologically normal and unactivated on light microscopy and, when compared with unfixed platelets, had comparable levels of binding to monoclonal antibodies directed against glycoproteins Ib, IIb, and FcγRII, as measured by flow cytometry. Moreover, fixed platelets minimally bound monoclonal antibodies to GMP-140 (P-selectin), further indicating that they had not been activated (4).

Platelet monolayers were prepared by placing 10^8 fixed platelets into poly-L-lysine-treated, 35-mm-diameter tissue culture wells. After 30 min at 37°C, the unbound platelets were removed by aspiration. To reduce nonspecific bacterial adherence, the wells were then incubated with 1% casein in Tyrode's solution for 1 h and washed.

To assay bacterial binding to immobilized platelets, overnight cultures of staphylococci in BHI broth and the appropriate antibiotic were washed twice in TEN buffer (0.1 M NaCl, 0.05 M Tris-HCl, 0.02 M EDTA [pH 7.25]) (25), suspended in Tyrode's solution, and sonicated for 10 s to disperse clumps (sonication had no effect on viability). The final concentrations were adjusted to 10^8 bacteria per ml, as determined by counting in a hemacytometer, and confirmed by plating on BHI agar. One milliliter of each bacterial suspension was added to individual wells in duplicate, and the plates were then incubated at 4°C, with gentle rocking. At preselected time points (15 min, 1 h, and 2 h), the wells were washed three times to remove unbound bacteria and then treated with trypsin (250 μg/ml; Clonetics, San Diego, Calif.) and scraped with a sterile spatula to release bound organisms (trypsin did not alter bacterial viability). The wells were then examined by microscopy to confirm that platelets had been lysed completely and that the bacteria had been released from the well bottoms. The number of organisms bound per well was determined by plating serial dilutions of the suspension onto BHI agar containing the appropriate antibiotic, and binding was expressed as the percentage of the inoculum (i.e., the total number of CFU placed in the wells) bound per well. As a control for nonspecific adherence, the binding of staphylococci to wells treated with poly-L-lysine and 1% casein, but lacking platelets, was also assessed in parallel. For some studies, bacteria were prepared as detailed above but treated for 1 h at 37°C with 1 mg of either trypsin (Sigma) or proteinase K (Sigma) per ml as described previously (24); addition of the appropriate protease inhibitors, washing, and testing for adherence to platelets followed. On microscopy, no reduction was seen in the confluency of platelet monolayers after exposure to enzyme-treated organisms, indicating that the bacterial suspensions had no residual proteolytic activity.

Flow cytometry. The binding of *S. aureus* to human platelets was also assayed by flow cytometry, as described previously (7). Bacteria and platelets were labelled internally with Hoechst 33342 (Molecular Probes, Eugene, Oreg.) and 5 μM 5-chloromethylfluorescein diacetate (Molecular Probes), respectively, and then washed and suspended in Tyrode's solution. Binding studies were performed at 20°C by mixing bacteria and platelets in Tyrode's solution at a final ratio of 10:1. One minute after mixing, the suspensions were analyzed by flow cytometry with a FACStarPlus cytofluorograph (Becton Dickinson, San Jose, Calif.), two argon lasers (Coherent, Mountain View, Calif.) operating at 150 mW, and the appropriate combinations of excitatory wavelengths and filters (24). The percentage of bacteria bound to platelets was determined by dividing the number of dually-labelled particles (representing bacteria bound to platelets) by the number of particles labelled with Hoechst 33342 (i.e., the total number of bacteria in suspension) and multiplying by 100.

Transposon mutagenesis and selection for low-binding variants. The *S. aureus* parent strain ISP479 carries the thermosensitive plasmid pI258, which can be used to deliver the transposon Tn*551* to the staphylococcal chromosome (21). Transposon mutagenesis of ISP479 was performed by growing the strain for 18 h at 32°C in BHI broth containing ampicillin and erythromycin. The culture was then diluted 1:2,000 in BHI broth containing erythromycin and incubated at 43°C for 24 h. The resultant organisms were washed twice in TEN buffer, suspended in phosphate-buffered saline, sonicated for 10 s, and adjusted to a final concentration of 5×10^9 bacteria per ml, as measured by counting in a hemacytometer. To enrich for low-binding isogenic mutants, 1 ml of the suspension was then centrifuged at 4°C onto human platelets immobilized in six-well tissue culture plates ($100 \times g$, 10 min). The plates were vortexed to resuspend nonadherent organisms, which were then collected and passaged again over immobilized platelets. After 12 passages, the remaining bacteria were plated onto BHI agar containing erythromycin and incubated for 18 h at 43°C.

The resultant colonies (representing candidate low-platelet-binding clones) were tested individually for a reduction in platelet binding. As an initial screening procedure, we used a modified version of the semiquantitative assay described previously by Nizet et al. (19). Overnight cultures of each colony (in BHI broth containing erythromycin) were washed as described above and suspended in Tyrode's solution. One hundred microliters of each bacterial suspension (10^7 CFU) were placed onto platelets immobilized in 96-well microtiter plates as described above and rocked gently at 4°C for 1 h. The unbound organisms were then removed by washing three times with Tyrode's solution. The wells were treated with trypsin (1 mg/ml, 10 min) to release the adherent bacteria, and then 175 μl of 0.7% Bacto Agar (Difco) containing 15 μg of erythromycin per ml was added. After overnight incubation at 32°C, the number of organisms per well was assessed qualitatively by visual inspection. Clones estimated to have at least 50% less binding than that of the parent strain represented possible low-binding isogenic mutants. These clones were subsequently tested individually by the more quantitative binding assay described above. The total number of CFU and the percentage of the inoculum bound were determined for each clone and compared with the binding values for ISP479 or ISP479C. The latter strain was used to confirm that binding was unaffected by the presence of pI258 or by growth in erythromycin.

Southern blot hybridization. *S. aureus* chromosomal DNA was prepared from lysostaphin-treated cells as described previously (6), digested with selected restriction enzymes (New England BioLabs, Beverly, Mass., or Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and transferred onto a Hybond N+ membrane (Amersham, Arlington Heights, Ill.). Hybridization was performed at 65°C with α-^{32}P-labelled or digoxigenin-labelled probes (DIG/Genius System; Boehringer Mannheim), followed by washing and imaging by autoradiography or chemiluminescence as described in the manufacturer's instructions.

Transduction. Phage ϕ11 lysates of strain PS12 were prepared as described previously (20). Generalized transduction of strains ISP479C and Newman was performed (7) at a low multiplicity of infection (phage-to-recipient ratio, 1:10). Transductants were selected on 0.3 GL agar containing erythromycin.

Phenotypic characterization. Isogenic strains of *S. aureus* were compared by the following characteristics: hemolysis on sheep blood agar, lipase production, protein A expression, and susceptibility to antimicrobials and cadmium at the concentrations described above (7). Growth rate at 37°C in BHI broth was assessed spectrophotometrically at 600 nm, over an optical density range of 0.05 to 1.0. Additional phenotypic characteristics were evaluated with the Vitek Gram-Positive Identification Card (bioMérieux, Hazelwood, Mo.). Binding to soluble fibrinogen and fibronectin binding were assessed by using ^{125}I-labelled proteins (7). In vitro susceptibility to the staphylocidal action of thrombin-derived platelet microbicidal protein (t-PMP) was measured as described previously (27), with an inoculum of 10^3 CFU/ml and a final t-PMP concentration of 1 μg/ml. *Bacillus subtilis* ATCC 6633 (a highly t-PMP-susceptible organism) and ISP479R (a highly t-PMP-resistant variant of ISP479 [27]) served as controls for t-PMP susceptibility and resistance, respectively. As in previous studies, the breakpoint for in vitro resistance of gram-positive bacteria to t-PMP was defined as ≥40% survival after 2 h of exposure (9, 26).

Northern (RNA) blot hybridization. Transcription of the staphylococcal global regulatory loci *agr* and *sar* were examined by Northern blotting. *S. aureus* RNA

was prepared with the FastRNA isolation kit (Bio 101, Vista, Calif.) in combination with 0.1-mm-diameter zirconia-silica beads and a FastPrep reciprocating shaker (Bio 101) as described previously (13). Ten micrograms of each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in MOPS (morpholinepropanesulfonic acid) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA [pH 7.0]). Blotting of RNA onto a nylon membrane (MSI Inc., Westborough, Mass.) was performed with the TurboBlotter Alkaline Transfer System (Schleicher & Schuell, Inc., Keene, N.H.). For detection of *agr* and *sar* transcripts, gel-purified DNA probes were radiolabelled with [α-^{32}P]dCTP (Amersham) by the random primer method (Ready to Go Labeling Kit; Pharmacia, Piscataway, N.J.) and hybridized under high-stringency conditions. The blots were subsequently autoradiographed.

Platelet aggregometry. The ability of staphylococci to induce platelet aggregation in vitro was evaluated as described previously (2). In brief, aggregometry was performed by adding 50 μl of staphylococci suspended in Tyrode's solution to 450 μl of platelet-rich plasma (final bacterium/platelet ratio, 5:1). Platelet aggregation was detected as an increase in light transmission, with platelet-poor plasma serving as a standard for 100% light transmission. Platelets were also tested with 10 μM ADP (Sigma) to ensure the responsiveness of each preparation. Strains were compared with regards to the interval between the addition of organisms to the platelet suspension and the onset of aggregation (lag time), rate of aggregation (slope at the midpoint of aggregation), and maximal change in light transmission. All strains were tested on multiple occasions with platelets from different donors or animals.

Animal model of endocarditis. Inocula were prepared from overnight cultures of strains ISP479C and PS12 that had been washed twice and suspended in sterile normal saline to an optical density at 620 nm of 1.6 ($\sim 10^9$ CFU). This suspension was serially diluted in saline to concentrations of 10^3 to 10^6 CFU/ml, as measured by plating onto blood agar.

Endocarditis of the aortic valve was produced in New Zealand White rabbits (2 to 2.5 kg) as described previously (11). In brief, animals were anesthetized with ketamine chloride (35 mg/kg, intramuscularly) and xylazine (1.5 mg/kg, intramuscularly). Sterile thrombotic endocarditis was produced by the placement of a polyethylene catheter in the left ventricle via the right carotid artery. Groups of 10 animals each were challenged intravenously 48 h after catheterization with strain ISP479C or PS12 at a range of inocula (10^3 to 10^6 CFU) that encompassed the 90% infective dose for inducing infective endocarditis by most *S. aureus* strains. Forty-eight hours after injection of bacteria, the rabbits were sacrificed with a rapid intravenous injection of sodium pentobarbital (100 mg/kg). Only animals with macroscopic valvular vegetations and proper catheter placement across the aortic valve were evaluated further. The aortic valves and left ventricular vegetations from individual animals were removed, pooled, weighed, homogenized in 0.5 ml of normal saline, and cultured quantitatively in the appropriate medium. For each challenge inoculum, differences in the density of organisms within vegetations of ISP479C- or PS12-infected animals were analyzed by two methods. First, the mean values of $\log_{10}$ CFU per gram of vegetation were compared for all rabbits within each group; animals with negative cultures of their vegetations were considered to have 2 $\log_{10}$ CFU/g (the lower limit of detection). A second analysis was then performed, comparing the mean values (± standard deviations [SDs]) of $\log_{10}$ CFU per gram of vegetation for each challenge inoculum, by using data only from animals with culture-positive vegetations.

To assess the incidence and extent of peripheral embolization during the course of endocarditis, both kidneys were harvested from all animals at the time of sacrifice and inspected for macroscopic evidence of abscess formation (1). If present, abscesses were excised, weighed, homogenized, pooled, and cultured quantitatively for each animal as described above. If no macroscopic lesions were visible, a random wedge tissue biopsy (sample size, approximately 1 cm^3) was performed on each kidney, and the tissue samples were processed identically. For each inoculum tested, the proportions of animals in each group with renal seeding were compared, as were the densities of organisms (CFU per gram) within the renal tissue. Culture-negative samples were considered to contain less than 2 $\log_{10}$ CFU/g, the lower limit of detection.

To determine the relative abilities of ISP479C and PS12 to adhere in vivo to the valve surface, catheterized rabbits were challenged intravenously with 8×10^6 CFU of either strain. Thirty minutes later, the animals were sacrificed, and vegetations were harvested and cultured quantitatively. To ensure that any observed differences in valvular adherence was not due to a disparity in achievable levels of bacteremia or rates of bacteremic clearance, quantitative blood cultures were obtained 1 min and 30 min after inoculation.

Statistical analysis. Differences between two strains in platelet binding were compared by the unpaired *t* test. Platelet binding between multiple strains was assessed by analysis of variance and the Newman-Keuls test. The abilities of ISP479C and PS12 to induce endocarditis or renal seeding were assessed by the Fisher exact test. Differences in vegetation or renal titers were compared by the Kruskal-Wallis test or the unpaired *t* test.

RESULTS

Binding of *S. aureus* to human platelets in vitro. Strain ISP479 readily bound to platelets immobilized in tissue culture wells. The number of CFU per well bound to platelets at 1 h (426,705 ± 229,450 [mean ± SD]) was significantly greater than that observed with tissue culture wells lacking platelets (78,485 ± 51,424; $P < 0.001$, $N = 17$). When expressed as a percentage of the inoculum, the binding of ISP479 to platelets at 1 h remained significantly higher than background (mean ± SD = 0.5% ± 0.24% versus 0.06% ± 0.03%; $P < 0.001$). As compared with ISP479 binding, similar levels of binding to platelets were observed with strain ISP479C (338,454 ± 142,768 CFU per well; 0.39% ± 0.20% inoculum bound; $N = 11$, P = not significant). Adherence of staphylococci was time dependent; after 15 min of incubation, 0.28% ± 0.04% of the inoculum had bound to platelets. No significant increase in binding was detected at time points beyond 1 h. Binding was also protease sensitive, as preexposure of ISP479 to trypsin or proteinase K reduced binding by 55.1% ± 5.6% and 69.9% ± 4.3% ($P < 0.02$), respectively.

Transposon mutagenesis and isolation of mutant PS12. ISP479 was cultured at 32 and 43°C as described above and then passaged repeatedly over immobilized platelets, to enrich for low-binding variants, and plated onto BHI agar containing erythromycin. The resultant colonies were screened further for a reduction in platelet binding, by using the qualitative and quantitative assays described above, leading to the identification of mutant PS12. When tested in parallel with ISP479, the percentage of the PS12 inoculum bound to immobilized platelets at 1 h (0.12% ± 0.05%) was 67.2% ± 8.3% less than that observed with ISP479 (0.37% ± 0.15%; $P < 0.001$, $N = 10$).

The binding of platelets in suspension by PS12 was also markedly reduced, as compared with that of the parental strain. One minute after mixing ISP479 and washed platelets in Tyrode's solution, 38.7% ± 8.6% ($N = 6$) of the bacteria in the suspension were bound to platelets, as measured by flow cytometry. Comparable levels of binding were seen with ISP479C. However, when PS12 was tested, only 11.1% ± 5.8% of the organisms were bound to platelets at this time point ($P < 0.001$, as compared with ISP479), a 71.3% reduction in binding. No significant increase in binding by either strain was seen at 30 or 60 min after mixing.

Confirmation of transposon insertion by Southern blotting. Since Tn*917* has significant homology with Tn*551*, we used a gel-purified 1.6-kb *Hin*dIII fragment of Tn*917* to probe Southern blot digests of PS12 chromosomal DNA. As shown in Fig. 1, three bands hybridized with the probe. Since Tn*551* contains two internal *Hin*dIII sites (5), these results indicate that a single copy of this transposon was inserted within the chromosome of PS12. Similar results were also obtained when we used a 5.6-kb *Eco*RI fragment of pLTV1 as a probe, which encompassed the entire Tn*917* sequence. As expected, only one hybridizing band was found, since Tn*551* does not contain an internal *Eco*RI site (5).

Phenotypic characterization of PS12. When compared with ISP479 and ISP479C, PS12 was unchanged with respect to numerous phenotypic properties. In particular, hemolytic activities, lipase production levels, protein A expression levels, and rates of growth in vitro were comparable. Moreover, these strains were identical when tested for 30 different metabolic characteristics with the Vitek Gram Positive Identification Card. ISP479, ISP479C, and PS12 were then tested for sensitivity to ampicillin, erythromycin, and cadmium. As expected, ISP479 was resistant to all three agents, indicating the retention of pI258, while ISP479C was fully susceptible, consistent with the absence of this plasmid. Strain PS12 was sensitive to cadmium and ampicillin but resistant to erythromycin, indicating that Tn*551* had been retained via chromosomal integration, with the concomitant loss of pI258.

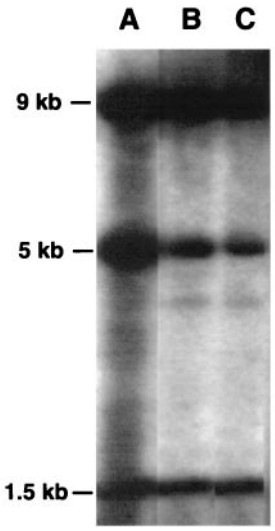

FIG. 1. Southern blots of PS12 and transductants of Newman. *Hin*dIII chromosomal digests of PS12 (A), Newman transductant I (B), and Newman transductant J (C) probed with a 1.6-kb *Hin*dIII fragment of Tn*917*. Since Tn*551* contains two internal *Hin*dIII sites, three fragments are expected from a single transposon insertion.

Effect of transposon insertion on binding to fibrinogen, fibronectin, platelet aggregation, and t-PMP sensitivity. Since fibrinogen and fibronectin binding have been associated with staphylococcal virulence in animal models of endocarditis (7, 8, 17), we compared the relative binding levels of these proteins by ISP479C and PS12. The amount of fibrinogen bound by 10^9 CFU of PS12 (2,032 ± 355 cpm [mean ± SD]) was comparable to that bound by ISP479C (2,378 ± 369 cpm; $N = 3$, P = not significant). PS12 and ISP479C also had similar levels of fibronectin binding (15,463 ± 2,897 and 17,911 ± 1,424 cpm, respectively; $N = 3$, P = not significant).

In addition, we compared the abilities of these strains to induce platelet aggregation in vitro. When tested with human platelets, all three strains induced irreversible aggregation. As compared with that of ISP479 and ISP479C, however, platelet aggregation by PS12 had a consistently longer lag phase for all donors tested ($N = 5$). More-pronounced differences were seen when the organisms were tested against rabbit platelets (Fig. 2). ISP479 and ISP479C produced biphasic aggregation, as described previously. In contrast, PS12 induced only the first phase of platelet aggregation, followed by disaggregation.

Since resistance to t-PMP may result in increased virulence in endocarditis (9, 26), we compared the susceptibilities in vitro of ISP479 and PS12 to t-PMP. After exposure for 2 h at 37°C to 1 μg of t-PMP per ml, the percent survival values of ISP479 and PS12 were 8% ± 3.5% and 2% ± 1.2% (mean ± SD), respectively (P = NS). In control studies, the percent survival values of *B. subtilis* ATCC 6633 and *S. aureus* ISP479R were 0% ± 0% and 51% ± 15.0%. Thus, strains ISP479 and PS12 were comparably susceptible to the microbicidal activity of t-PMP.

Transduction of low-platelet-binding phenotype. To confirm that the loss of platelet binding was linked to gene interruption by Tn*551*, we transduced the mutation associated with PS12 into strains ISP479C and Newman. Colonies resistant to erythromycin were then evaluated for adherence to platelet monolayers. As compared with that of their respective parent strains, platelet binding levels by transductants derived from strains ISP479C and Newman were significantly reduced. In particular, the percentages of the inoculum bound by ISP479C transductants A and B (0.36% ± 0.20% and 0.22% ± 0.18%, respectively) were significantly less ($P < 0.05$) than that observed with the parent strain (0.72% ± 0.31%). Similarly, platelet binding levels found for Newman transductants I and J (0.32% ± 0.16% and 0.20% ± 0.08%, respectively) were significantly less ($P < 0.05$) than that seen with the parental strain (0.64% ± 0.10%; $P < 0.05$). Southern blot analysis of chromosomal DNA purified from these transductants confirmed that a single copy of Tn*551* was contained within their genomes (Fig. 1). The pattern of hybridizing bands for the transductants indicated that the transposon was likely to have inserted into a chromosomal location identical to that of the mutant PS12.

Northern blot analysis for *agr* and *sar* expression. Two major loci for the global regulation of virulence determinants have been described in *S. aureus* and termed *agr* and *sar* (7, 8, 16). To assess whether the reduction in platelet binding seen with PS12 was due to inactivation of these loci, we examined *agr* and *sar* expression by Northern blotting. When probed with a PCR-derived 513-bp *agrA* fragment to assess transcription of RNAII (a 3.5-kb transcript within the *agr* system), PS12 was found to have RNAII levels comparable to that of the parental

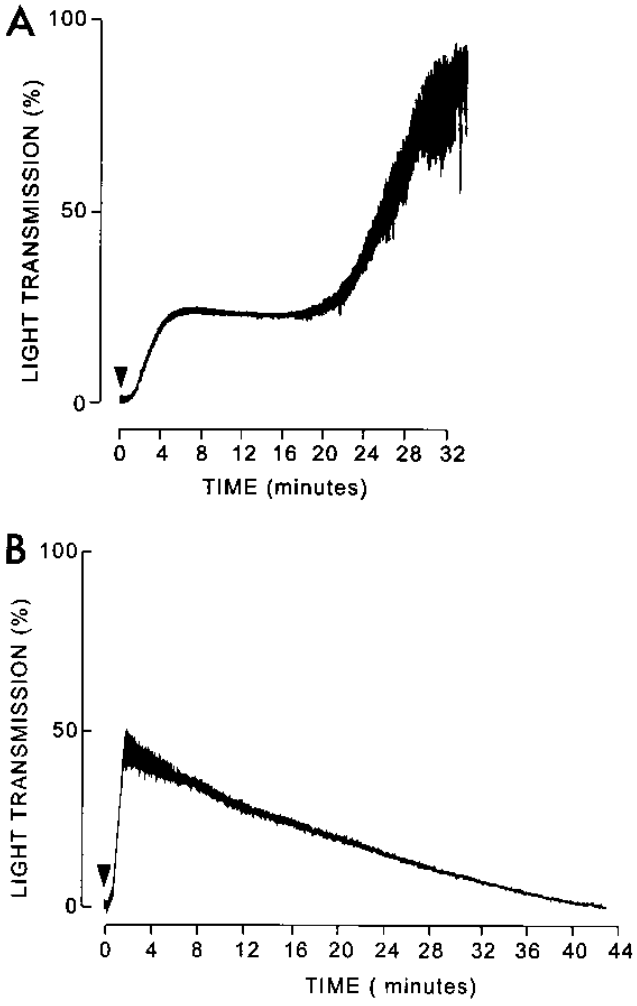

FIG. 2. Aggregation of rabbit platelets by ISP479 (A) and PS12 (B). Arrowheads indicate the times when staphylococci were added to platelets. Note the biphasic, irreversible aggregation by ISP479. In contrast, PS12 induced only the first phase of aggregation, followed by disaggregation.

TABLE 2. Virulence of ISP479C versus PS12 in the rabbit model of endocarditis

Strain	Inoculum (CFU)	Endocarditis induction rate[a]	Vegetation titer[b]	Renal seeding[c]	Renal titer[b]
ISP479C	10^3	5/11 (45)[d]	3.10 ± 0.4	5/11 (46)[d]	2.69 ± 0.98
	10^4	10/10 (100)	5.36 ± 2.0	7/10 (70)	2.79 ± 0.78
	10^5	11/11 (100)	6.23 ± 2.3	11/11 (100)	3.99 ± 1.58
	10^6	9/9 (100)	5.80 ± 2.9	9/9 (100)	5.64 ± 1.6
PS12	10^3	0/10[e] (0)	≤2.0 ± 0[e]	0/10[e] (0)	≤2.0 ± 0[e]
	10^4	0/10[e] (0)	≤2.0 ± 0[e]	0/10[e] (0)	≤2.0 ± 0[e]
	10^5	4/10[e] (40)	4.50 ± 1.9	0/10[e] (0)	≤2.0 ± 0[e]
	10^6	9/10 (90)	6.23 ± 1.6	7/10[e] (70)	4.2 ± 1.4[e]

[a] Number of animals developing infective endocarditis/total number inoculated.
[b] Log_{10} CFU per gram (mean ± SD).
[c] Number of animals developing metastatic foci of infection/total number inoculated.
[d] Values in parentheses are percentages.
[e] $P \leq 0.05$, ISP479 versus PS12, for the same inoculum.

strain. Similarly, probing with a 732-bp *sar* probe revealed levels of *sar* expression by PS12 that were comparable to those observed with ISP479C. *sar* and *agr* expression levels in strain Newman and its low-binding transductant, mutant I, were also comparable, confirming that these loci were unaffected by Tn*551* insertion.

Animal model of endocarditis. The abilities of ISP479C and PS12 to induce endocarditis were compared by using a range of inocula (Table 2). For inocula of 10^3, 10^4, and 10^5 CFU, the rates of induction for PS12 were significantly lower than those seen with the parent strain (0, 0, and 40% versus 45, 100, and 100%, respectively; $P = 0.035$, 0.001, and 0.041, respectively). At the highest inoculum tested (10^6), no significant difference in rates of induction was observed.

The density of organisms within vegetations also differed between ISP479C and PS12 for inocula of 10^3, 10^4, or 10^5 CFU. When values from all animals were analyzed (including those with sterile vegetations), the mean number of $\log_{10}$ CFU per gram was significantly less for PS12, as compared with that of ISP479 (Table 2). Data for the two strains were then reanalyzed, by using only values from the animals with positive vegetation cultures. Since none of the animals inoculated with 10^3 or 10^4 CFU of PS12 developed endocarditis, this analysis could be done only for the animals receiving the larger inocula. At an inoculum of 10^5 CFU, the vegetation densities for PS12 were lower than those for ISP479, but this difference was not statistically significant. No significant difference was seen at the highest inoculum (10^6 CFU).

Animals inoculated with PS12 were significantly less likely to develop septic embolization to the kidneys (Table 2). At inocula of 10^3, 10^4, and 10^5 CFU, the percentages of animals with renal abscesses were significantly lower in the PS12 groups (0, 0, and 0%, respectively) than in the ISP479C groups (46, 70, and 100%; $P = 0.035$, 0.031, and 0.00003, respectively). In addition, the density of organisms (CFU per gram) within renal abscesses was significantly lower in PS12-infected animals over the entire range of inocula, including 10^6 CFU.

To verify the stability of Tn*551* insertion after animal passage, chromosomal DNA was extracted from PS12 colonies recovered from the vegetations of three rabbits, digested with *Eco*RI, and probed by Southern blotting with a 5.3-kb *Eco*RI fragment of pLTV1 that encompassed Tn*917*. For all three colonies tested, a single, 16-kb hybridizing band, similar to that seen with PS12 before passage, was noted. These results indicate that the transposon remained stably integrated within the chromosome of PS12 during the course of endocardial infection.

To determine whether the disparities described above in endocarditis induction rates were due to differences in the initial binding of organisms to the valve surface, we sacrificed separate groups of catheterized animals 30 min after intravenous challenge with 10^6 CFU of either ISP479C or PS12. Quantitative blood cultures also were obtained 1 and 30 min after inoculation. The number of CFU per vegetation for ISP479C (69.2 ± 44 [mean ± SD]; $N = 5$) was not significantly different from that observed with PS12 (91.5 ± 67; $N = 6$). Similarly, no differences were observed in the levels of bacteremia between the two strains at 1 or 30 min after intravenous challenge.

DISCUSSION

To address the role of platelet binding by bacteria in the pathogenesis of endocarditis, we sought to derive an isogenic mutant of *S. aureus* ISP479 that differed only in its ability to bind platelets directly (i.e., in the absence of plasma components or other potential bridging molecules). By means of transposon mutagenesis, we generated strain PS12; as compared with that of its parent strain, binding in vitro of PS12 to immobilized platelets was markedly reduced. In addition, analysis by flow cytometry showed that the ability of PS12 to bind platelets in suspension was significantly less than that seen with ISP479. This latter observation is important for two reasons. First, it confirms the low-platelet-binding phenotype of PS12 by a methodology distinct from that used originally for the enrichment and selection of this strain. Second, it demonstrates that the mutation in PS12 alters binding to platelets regardless of whether these cells are in suspension or immobilized on a surface. This suggests that in vivo, staphylococci may bind to platelets under both conditions.

Apart from differences in platelet binding, ISP479 and PS12 were phenotypically identical, as measured by numerous in vitro assays, including the expression of two endocarditis-related virulence factors, fibrinogen and fibronectin binding. In addition, the expression levels of two major virulence-related regulons (*agr* and *sar*) were comparable in these strains. Thus, Tn*551* insertional mutagenesis appears to have reduced platelet binding selectively. That the loss of platelet binding was due to Tn*551* insertion was confirmed by our transduction studies, in which the low-binding phenotype was transferred successfully into strains ISP479C and Newman.

Of note, the aggregation of platelets in vitro by strain PS12 differed substantially from that of its parent. With human platelets, the onset of PS12-induced aggregation was delayed, suggesting that, although not critical for aggregation, direct binding of staphylococci to platelets may facilitate the formation of platelet aggregates. When PS12 was tested with rabbit platelets, the first phase of aggregation (which is coincident with platelet activation) was unaltered. In contrast to aggregation by ISP479, however, PS12 failed to induce the second phase of platelet aggregation. On the basis of our previous studies of platelet aggregation by *S. aureus*, we had postulated that this second phase requires the direct binding of staphylococci and platelets, followed by fibrinogen-mediated cross-linking of these bacterium-platelet complexes (2). This model is supported by our findings with PS12, since the reduced level of direct platelet binding seen with this organism would mitigate the subsequent formation of aggregates, despite an intact ability to bind fibrinogen in vitro.

When tested in an animal model of endocardial infection,

PS12 proved to be markedly less virulent. As compared with strain ISP479C, rabbits inoculated with 10^3 to 10^5 CFU of PS12 were significantly less likely to develop endocarditis. Moreover, animals infected with PS12 had significantly lower densities of organisms (CFU per gram) within vegetations. In addition, PS12-infected rabbits had a markedly lower incidence of metastatic seeding to the kidneys as well as lower concentrations of organisms within the renal parenchyma. Thus, our results indicate that the direct binding of *S. aureus* to platelets is a major determinant of microbial virulence in the pathogenesis of endocarditis. This binding appears to be important both for development of endocardial infection and for its embolic complications.

The mechanisms by which bacterium-platelet binding may enhance pathogenicity are unknown. It has been suggested that platelets on the surface of damaged valves may facilitate the initiation of infection by serving as binding sites for circulating microorganisms (15, 22). However, in our studies of rabbits sacrificed 30 min after inoculation, no difference was seen in the densities of ISP479C and PS12 on damaged valve surfaces, suggesting that these strains had comparable levels of initial binding. This is not entirely surprising, since our previous studies indicate that the initial binding of *S. aureus* to the endocardium is mediated predominantly by *agr*- and *sar*-dependent mechanisms, such as the expression of receptors for fibrinogen and fibronectin (7, 8). Thus, our data suggest that direct staphylococcus-platelet binding may be more important for pathogenetic events that occur after the initial attachment of organisms to the valve surface. It has been postulated that the continuous deposition of platelets onto infected valves produces a physical barrier to the clearance of organisms by host defenses (22). However, studies attempting to validate this hypothesis have yielded conflicting results (18, 22). Another possibility is that bacterium-platelet binding is critical for endocardial reseeding, i.e., the shedding of bacteria from the valve into the bloodstream, followed by their reattachment to the endocardium. Only indirect, morphologic evidence exists for reseeding in vivo (10). In view of the continuous bacteremia that is a hallmark of endocarditis, however, it is likely that, as platelets accumulate at the site of infection, reattachment of organisms to the valve surface becomes increasingly mediated by platelet binding, leading to the propagation and evolution of the vegetation.

Systemic embolization with secondary infection of extracardiac organs is one of the most frequent complications of infective endocarditis. Septic emboli are thought to arise from platelet-fibrin fragments of the vegetation that break off from the valve surface, thereby entering the circulation. The reduced incidence of renal embolization in PS12-infected animals may reflect changes in vegetation structure secondary to decreased platelet binding and subsequent aggregation, such that either the vegetations are less friable or the vegetation fragments released into the bloodstream are less likely to become lodged within the circulation. The latter may be due simply to a reduction in size of the emboli. Alternatively, the reduced platelet content of these fragments may render them less capable of attaching to the endothelial surface of the microvasculature.

As for the molecular basis of staphylococcal binding to platelets, our results indicate that adhesion is mediated by one or more trypsin-sensitive ligands on the bacterial surface. The reduced platelet binding observed with strain PS12 may be due to a mutation of either a structural or a regulatory gene involved in the expression of such ligands. Studies are now in progress to identify this gene and its product as well as to characterize possible platelet receptors for staphylococcal ligands.

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