Phenotypic Resistance to Thrombin-Induced Platelet Microbicidal Protein In Vitro Is Correlated with Enhanced Virulence in Experimental Endocarditis Due to *Staphylococcus aureus*

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Thrombin-induced platelet microbicidal protein (tPMP) is secreted by rabbit platelets following thrombin stimulation, and it kills common endovascular pathogens in vitro, including *Staphylococcus aureus*. Therefore, pathogens which exhibit tPMP resistance in vitro possess a potential survival advantage in vivo at sites of endovascular damage. We generated an isogenic *S. aureus* strain pair, differing in tPMP susceptibility, by transposon (Tn551) mutagenesis of a tPMP-susceptible (tPMP^s) parental strain (ISP479) to derive a stably tPMP-resistant (tPMP^r) strain, ISP479R. ISP479 and ISP479R were equivalent in vitro in the following phenotypes: biotyping, antibiograms, platelet adherence and aggregation, growth kinetics, cell wall-associated protein A expression, and fibrinogen binding. Genotypic comparisons of chromosomal DNA of strains ISP479 and ISP479R following restriction endonuclease digestion revealed indistinguishable pulsed-field gel electrophoretic patterns. The genotype exhibited by strain ISP479R was linked to the tPMP-resistant phenotype, as it was transducible into the initially tPMP-susceptible parental strain, ISP479. Southern hybridization verified the presence of a single copy of Tn551 in the same chromosomal restriction site of both ISP479R and tPMP^r transductants of ISP479. The correlation of in vitro tPMP susceptibility phenotypes with the ability to induce experimental endocarditis (a prototypical endovascular infection) was evaluated. Despite equivalent rates of endocarditis induction, animals infected with strain ISP479R achieved significantly higher vegetation bacterial densities over a 7-day post-challenge period than did animals infected with strain ISP479. These data suggest that tPMP^r microbial strains have a selective advantage in experimental staphylococcal endocarditis. Furthermore, the major impact of tPMP resistance upon endocarditis pathogenesis appears to involve a postvalvular adherence event(s), most probably by facilitating bacterial proliferation within vegetations.

The pathogenesis of infective endocarditis is a complex process, involving at least four sequential host-pathogen interactions: (i) endocardial damage, inducing platelet and fibrin deposition at this site (18); (ii) adherence of bloodstream pathogens to damaged endocardial sites (11, 28); (iii) further deposition of platelets and fibrin onto the damaged, infected endocardium (28); and (iv) endocardial reseeding, either hematogenously from distant extracardiac abscesses or locally by intraendocardial microorganisms (1, 10, 26). Platelets have been traditionally viewed as contributing to the induction and progression of the vegetative lesion in endocarditis (16, 17, 21). In contrast, recent evidence from our laboratory (33) and others (7) has indicated that platelets also play an important host defense role, serving to limit the propagation of endocarditis. Our in vitro studies have shown that thrombin (a molecule generated at sites of endocardial damage in vivo [8, 9]) elicits the secretion from rabbit platelets of a low-molecular-weight cationic protein with potent antimicrobial properties, termed thrombin-induced platelet microbicidal protein (tPMP [32]). This peptide exhibits microbicidal activity in vitro against common endovascular pathogens, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, viridans group streptococci, and

Candida albicans (20, 29, 32). Of note, most strains isolated from blood cultures of patients with endocarditis exhibit resistance to tPMP in vitro, while those from patients without endocarditis are largely susceptible to tPMP (29). The present study was designed to directly examine the in vivo influence of in vitro tPMP susceptibility or resistance upon the induction and progression of endocarditis due to *S. aureus* in a well-established animal model.

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MATERIALS AND METHODS

tPMP. tPMP was prepared as previously described (32, 35). In brief, blood from New Zealand White rabbits was collected into siliconized tubes containing citrate anticoagulant. Anticoagulated whole blood was then centrifuged ($75 \times g$ for 10 min) to produce a platelet-rich plasma (PRP) supernatant containing <1% leukocyte contamination. The platelets were pelleted by centrifugation ($1,000 \times g$ for 10 min) of the upper two-thirds of the PRP supernatant, and the resulting platelet pellet was washed twice in Tyrode's salts solution (Sigma Chemical Co., St. Louis, Mo.) and resuspended in Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.). Preparations rich in tPMP were subsequently produced from washed platelet suspensions (10^8 /ml) by stimulation with bovine-derived thrombin (Sigma Chemical Co.) (1 U/ml at 37°C for 20 min) in the presence of 0.2 M CaCl₂. Following thrombin-induced platelet activation, residual platelet material was removed by centrifugation and the tPMP-rich supernatant was recovered. Substantial purification (approximately 25-fold, $\geq 80\%$ purity) of tPMP was achieved by anionic (cellulose acetate-nitrate) affinity adsorption and cationic elution as previously described (32). Preparations rich in tPMP were then pooled, dialyzed against 10 volumes of sterile deionized water (molecular mass cutoff, 3.5 kDa [Spectrapor 3; Spectrum Medical Industries, Los Angeles, Calif.]), and stored at -20°C .

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Bioactivity of tPMP preparations. The bioactivity of the tPMP preparations was determined by previously described methods (32). In brief, bioassays were performed with *Bacillus subtilis* (ATCC 6633), an indicator organism that is highly susceptible to the bactericidal action of tPMP (15). For the bioassay, *B. subtilis* was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37°C for 14 h, harvested by centrifugation, washed twice in 0.85% (wt/vol) sodium chloride (normal saline), and resuspended in phosphate-buffered saline (pH 7.2) prior to use. To determine tPMP bioactivity, *B. subtilis* at an inoculum of 10^4 CFU/ml was added to microtiter wells containing a range of dilutions of the tPMP-rich preparation to achieve a final inoculum of 10^3 CFU/ml per well and a final range of tPMP dilutions ranging from 1:1 to 1:1,024 (final well volume, 200 μ l). After 30 min of incubation at 37°C, a 20- μ l aliquot was removed from each well, diluted into phosphate-buffered saline containing 0.01% (wt/vol) sodium polyanethanol sulfonate (Sigma Chemical Co.) to inhibit further tPMP-induced bacterial killing, and quantitatively cultured onto 6.6% sheep blood agar. tPMP bioactivity (units per milliliter) was defined as the inverse of the highest tPMP dilution which retained $\geq 95\%$ lethality for *B. subtilis* within the 30-min assay period (32). The specific activity of each tPMP preparation was approximately 20 U/ μ g of protein as determined by spectrophotometry ($\lambda = 220$ nm).

***S. aureus* strains.** All staphylococcal strains were stored at -70°C until thawed for use. The organisms were routinely passaged in BHI broth overnight prior to use. After being harvested by centrifugation, the staphylococcal cells were washed and resuspended in normal saline, sonicated for 4 s to ensure singlet organisms, and adjusted spectrophotometrically (optical density at 600 nm [OD_{600}]) to the desired final inoculum. Bacterial inocula were routinely confirmed by quantitative cultures on BHI agar.

ISP479 (parental strain) is a tPMP-susceptible isolate (mean percent survival in vitro following tPMP exposure $\pm$ standard deviation [SD] = $14\% \pm 8\%$), which carries plasmid pI258, a temperature-sensitive replicon which encodes cadmium and ampicillin resistance (20). This plasmid contains transposon Tn551, which confers erythromycin resistance. ISP479C is a well-characterized *S. aureus* strain that is the spontaneous, plasmid-cured variant of parental strain ISP479 (26). ISP479C consistently induces experimental endocarditis (26) and has previously been used in studies of in vivo staphylococcus-platelet interactions in our laboratory (26). Its susceptibility to tPMP was determined by exposing 10^4 staphylococcal cells to 1 μ g of tPMP/ml for 2 h at 37°C in a microtiter well assay system, as previously described (32). Three independent runs on separate days were performed. As in prior studies, the breakpoint for in vitro resistance of gram-positive bacteria to tPMP was defined as $\geq 40\%$ survival after 2 h of exposure (29, 32). The mean ($\pm$ SD) percent survival of ISP479C was $8 \pm 3.5\%$ (26).

We constructed an isogenic tPMP^r variant of the tPMP^s parental strain, ISP479, by transposon mutagenesis after temperature shifting of the parental strain at 43°C to allow transposition to occur (26). tPMP^r mutants were derived by a three-stage method. The transposed population was first plated directly onto agar containing erythromycin (10 μ g/ml) and protamine sulfate (3 mg/ml), a small, cationic peptide (13, 33). The resistance of microbial strains to protamine correlates closely with resistance to tPMP and other endogenous antimicrobial peptides (13, 33). Thus, enrichment for protamine-resistant colonies is a useful surrogate screening marker for tPMP resistance. Moreover, the microbicidal actions of tPMP (but not of protamine) are neutralized in standard nutrient-based agars, making a tPMP-containing agar unfeasible for large-scale screening. Erythromycin-resistant and protamine-resistant variants were then evaluated for tPMP resistance by the microtiter assay described previously (32). tPMP-resistant transposon mutants were then assessed for loss of pI258 by being plated onto both ampicillin-containing (20 μ g/ml) agar and cadmium-containing (0.1 mM) agar (to confirm growth inhibition). A tPMP^r transposon mutant, ISP479R, was found to be erythromycin resistant and protamine resistant but ampicillin susceptible and cadmium susceptible. In addition, ISP479R exhibited stable tPMP^r in vitro after serial passage in the absence of tPMP (mean percent survival, $51\% \pm 15\%$ versus $8\% \pm 3.5\%$ for parental strain ISP479C). Moreover, Southern hybridization of strain ISP479R with a specific probe for Tn551 confirmed the presence of a single transposon chromosomal insert (see below). Strains ISP479C and ISP479R were therefore used in the remainder of the studies below.

Phenotypic comparison of the tPMP^s and tPMP^r *S. aureus* strains. A phenotypic comparison of ISP479C and ISP479R is given in Table 1. The ability of ISP479C and ISP479R to assimilate or metabolize a panel of carbohydrates was compared by using the Vitek gram-positive identification card (BioMerieux-Vitek, Inc., Hazelwood, Mo.) as specified by the manufacturer. In addition, the in vitro susceptibilities of these two strains to three standard antistaphylococcal agents, oxacillin, vancomycin, and gentamicin, were compared in a microtiter broth assay (23). Also, the two strains were compared for 24-h in vitro growth rates in BHI broth, as well as for in vitro resistance to killing by rabbit serum (22).

Strains ISP479C and ISP479R were indistinguishable in colony morphology, pigment (yellow), hemolysis pattern on sheep blood agar (β), and had nearly identical growth kinetics in BHI broth over a 24-h period (log and stationary growth phases achieved at 3 to 4 h and 7 to 8 h respectively). Moreover, both strains were coagulase positive, and were resistant to rabbit serum. Also, these strains were identical in carbohydrate metabolic profiles, and the MICs of oxacillin, vancomycin, and gentamicin for each strain were similar.

The capacity of staphylococci to bind to and aggregate platelets (16, 17, 24)

TABLE 1. Phenotypic comparison of ISP479C and ISP479R

Phenotypic trait	Value in:	
	ISP479C	ISP479R
Colony morphology	Mucoid; yellow	Mucoid; yellow
tPMP susceptibility (% survival at 2 h)	8 ± 3.5	51 ± 15
Hemolysis on 6.6% sheep blood agar	+ (clear)	+ (clear)
Coagulase	+	+
Oxacillin MIC (μ g/ml)	<0.125	<0.125
Vancomycin MIC (μ g/ml)	0.25	0.50
Gentamicin MIC (μ g/ml)	<0.125	<0.125
Mannitol fermentation	+	+
Sucrose fermentation	+	+
Raffinose fermentation	+	+
Dextrose fermentation	+	+
Fibrinogen binding (cpm/ 10^9 CFU $\pm$ SEM)	$5,926 \pm 589$	$4,810 \pm 415$

and to adhere to fibrinogen in vitro has been correlated with ability to induce experimental endocarditis (12, 19). Thus, we quantitatively compared ISP479C and ISP479R in terms of platelet binding and aggregation profiles, as well as fibrinogen binding. The binding of ISP479C and ISP479R to immobilized platelet monolayers was compared in a solid-phase assay as described by Sullam et al. (26). Platelet binding was expressed as the mean $\pm$ SD of the percentage of the initial staphylococcal inoculum ($\sim 2 \times 10^8$ CFU) which bound irreversibly to platelet monolayers (26). To compare the abilities of ISP479C and ISP479R to aggregate rabbit platelets in vitro, platelet aggregometry was performed as previously described, with a bacterium/platelet ratio of 10:1 (3). The binding of ISP479C and ISP479R to soluble fibrinogen was assessed with ^{125}I -labelled fibrinogen, as previously described (6). The binding of ISP479C and ISP479R to platelet monolayers was not significantly different (percent inoculum bound = 0.89 ± 0.22 and 1.32 ± 0.73 , respectively). Additionally, both strains induced aggregation of rabbit platelets in vitro, with nearly identical first- and second-phase aggregation profiles (reference 3) and data not shown). Both strains bound equally well in vitro to ^{125}I -labeled fibrinogen.

Cell wall-associated protein A is a predominant surface protein in *S. aureus*. This protein is believed to be an important virulence determinant, probably via its binding to host immunoglobulin G on damaged host tissues (5). The presence of protein A in lysostaphin-derived cell wall extracts of strains ISP479C and ISP479R was assayed on immunoblots, with affinity-purified chicken anti-protein A antibody as previously described (5). Immunoblots demonstrated equivalent expression of cell wall-associated protein A for strains ISP479C and ISP479R (data not shown).

Population analyses. We assessed whether the tPMP^s and tPMP^r phenotypes exhibited by strains ISP479C and ISP479R, respectively, were heterotypic or homotypic (14). As noted above, the bioactivity of tPMP is significantly reduced in solid nutrient agar media, preventing the use of tPMP-containing agar in quantitative population assays. As above, we have previously demonstrated that the in vitro susceptibilities of genetically related tPMP^s and tPMP^r microbial strains to the cationic antimicrobial peptide protamine closely mirrored those of tPMP (13). Therefore, protamine sulfate (Sigma) was used as a surrogate agent for tPMP in population analyses on solid agar (Luria-Bertani [LB] agar) with a final bacterial inoculum of $\sim 8 \log_{10}$ CFU/ml and final protamine concentrations of 0.125 to 10 mg/ml. After a 24-h incubation at 37°C, bacterial colonies were counted, with an end point being $>99.9\%$ growth inhibition and the shape of the population curve being used to define heterotypic versus homotypic susceptibility or resistance, as previously defined (14). Both strains exhibited homotypic population susceptibility patterns to protamine in vitro. Thus, complete growth inhibition of ISP479C and ISP479R by protamine was observed at 0.5 and 10 mg/ml, respectively, with no substantive growth inhibition seen at 0.375 and 9 mg/ml, respectively (Fig. 1). These data demonstrated that the tPMP^s and tPMP^r staphylococcal strains each probably represent homogeneous microbial populations with regards to tPMP susceptibilities.

Genotypic comparisons of tPMP^s and tPMP^r strains. The genotypic profiles of digested total DNA from strains ISP479, ISP479C, and ISP479R were compared by pulsed-field gel electrophoresis with a CHEF-DR II (Bio-Rad, Hercules, Calif.) (courtesy of Alan Hartstein, University of Indiana Medical Center, Indianapolis, Ind. [25]). Restriction endonuclease digestion profiles (with *smal*) of genomic DNA from these strains were evaluated by visual inspection of ethidium bromide-stained agarose gels following pulsed-field gel electrophoresis. Isolates were considered to be genotypically different when any readily detectable DNA bands did not migrate identically. Lambda phage concatemeric DNA (FMC Corp., Rockland, Maine) was routinely used for molecular size comparisons.

FIG. 1. Population dynamics of the isogenic *S. aureus* strain pair during exposure to the cationic antimicrobial peptide protamine (surrogate marker for tPMP).

Southern hybridization comparisons of tPMP^s and tPMP^r strains. Chromosomal DNA from *S. aureus* strains was prepared from lysostaphin-treated cells as described previously (4). DNA was digested with two restriction enzymes (*Eco*RI and *Hind*III [New England BioLabs, Beverly, Mass.]), electrophoresed, transferred onto Hybond N⁺ membrane (Amersham, Arlington Heights, Ill.), and hybridized with ³²P-labeled Tn917 probe as previously described (4); Tn917 shares >90% homology with Tn551. After hybridization, the membrane was washed with SSE (150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA [pH 7.4]) as previously described (4) and autoradiographed.

Transduction. To confirm that any chromosomal inserts identified on Southern hybridization of strain ISP479R were directly linked to the tPMP resistance phenotype exhibited by this strain, transduction studies were performed. Phage 11 lysates of strain ISP479R were prepared as previously described (20). Generalized transduction of the tPMP-susceptible parental strain, ISP479C, was performed (4) at a low multiplicity of infection (phage-to-recipient ratio, 1:10). Transductants were selected on 0.3 GL agar containing erythromycin. Candidate transductants were then assessed for in vitro tPMP resistance as above in the microtiter assay; tPMP^r candidates were then further evaluated by Southern hybridization to verify the presence of a single insertion of Tn551 into the chromosome of ISP479C at a site identical to ISP479R.

Rabbit model of endocarditis. The rabbit model of experimental endocarditis was used in these studies, as previously detailed (21). Briefly, anesthetized rabbits underwent transcarotid-transaortic valvular catheterization with an indwelling, polyethylene catheter to induce sterile valve vegetations. Staphylococcal endocarditis was produced by the intravenous (i.v.) injection of either strain ISP479C or ISP479R at 24 h postcatheterization. To examine the relative capacity of these two strains to induce endocarditis, separate groups of catheterized rabbits were challenged i.v. by a range of inocula of either staphylococcal strain (10³ to 10⁶ CFU) that encompasses the 95% infective dose of most *S. aureus* strains in this model (5, 26). To generate adequate statistical power, at least 11 catheterized rabbits were challenged with each staphylococcal strain, at all challenge inocula.

Early adherence of *S. aureus* to vegetations. In vivo adherence studies were performed to compare the relative abilities of the two staphylococcal strains to adhere to sterile cardiac vegetations, using the above endocarditis model. Two groups of rabbits underwent aortic catheterization as before and were challenged 24 h later with ~3 × 10⁶ or 3 × 10⁷ CFU of either *S. aureus* strain. In pilot studies in our laboratory, these inocula were shown to cause experimental endocarditis in 100% of catheterized rabbits challenged with either strain ISP479C or ISP479R. At 30 min after the i.v. challenge, the animals were sacrificed and all vegetations from individual animals were removed and quantitatively cultured. The in vivo adherence of each strain was expressed as the mean CFU adhering to sterile vegetations ± SD (4, 6).

Clearance of bacteremia. It is important to ensure that any differences observed in microbial ability to induce experimental endocarditis are not related to differences in the clearance of bacteremia after the i.v. challenge. Thus, blood samples were obtained from catheterized rabbits at 1 and 30 min after the challenge with either staphylococcal strain, for quantitative culture (4, 6).

Induction of endocarditis. The rabbits were sacrificed by a rapid i.v. injection of sodium pentobarbital (Abbott Laboratories, Chicago, Ill.) 24 h after the i.v. challenge with either strain ISP479C and ISP479R. At sacrifice, proper catheter position was verified and cardiac vegetations from individual animals were removed, weighed, pooled, homogenized, and quantitatively cultured as previously described (6). Intravegetation staphylococcal densities were expressed as log₁₀ CFU per gram. Endocarditis was considered to have been induced in any animal with culture-positive vegetations, irrespective of the final vegetation density.

Retention of tPMP^s and tPMP^r phenotypes in vivo. To confirm that strains ISP479C and ISP479R retained their individual tPMP susceptibility phenotypes during in vivo infection, separate groups of 21 catheterized rabbits were chal-

lenged with either staphylococcal strain. Three animals in each challenge group were sacrificed daily for seven consecutive days. At the time of sacrifice, the vegetations were processed as above. Vegetation homogenates were parallel-plated onto protamine-free LB agar, as well as onto protamine-containing LB agar. The final protamine concentrations used for culturing vegetation homogenates from animals infected with either staphylococcal strain were based upon growth-inhibitory data generated from the in vitro population study above (0.5 mg/ml for ISP479C; 10 mg/ml for ISP479R [Fig. 1]). In addition, colonies were selected from plates of protamine-free vegetation homogenates from animals infected with either staphylococcal strain and individually retested for retention of tPMP susceptibility or resistance in vivo by the microtiter assay described above.

Statistical analyses. Proportional differences in the frequencies of endocarditis induction were compared by Fisher's exact test. Differences in vegetation bacterial densities were compared by the Kruskal-Wallis test. *P* values of ≤0.05 were considered to be statistically significant.

RESULTS

Genotypic comparisons. Restriction endonuclease analyses with digests of chromosomal DNA revealed no major differences among the genotypes of ISP479, ISP479C, and ISP479R (Fig. 2).

Southern hybridization. Southern blotting of *Eco*RI-digested chromosomal DNA from mutant strain ISP479R produced a single band of hybridization (Fig. 3). Southern blotting of *Hind*III-digested DNA revealed two hybridizing bands (Fig. 3). Since there are no internal *Eco*RI sites within Tn551, this hybridization pattern indicates a single chromosomal transposon insert. Since Tn551 contains two internal *Hind*III restriction sites, three distinct hybridizing fragments would be predicted. However, only two bands hybridized with the Tn917 probe, suggesting that the third *Hind*III fragment may share limited homology with this probe. As predicted, Southern blots of parental strain ISP479C were negative.

Transduction of the tPMP^r phenotype. To verify that the induction of the tPMP^r phenotype in strain ISP479R was caused by gene inactivation mediated by Tn551, we transduced the mutation associated with ISP479R into the tPMP-susceptible parental strain, ISP479C. Following phage φ11 transduction and plating onto erythromycin-containing agar, erythromycin-resistant colonies were tested for tPMP^r in vitro. Of the first 25 clones tested, all 25 were tPMP^r (>90% survival after

FIG. 2. Pulsed-field gel electrophoresis of chromosomal DNA from strains ISP479C (lane 2), ISP479 (lane 3), and ISP479R (lane 4). The lambda phage control is in lane 1.

FIG. 3. Southern hybridization of chromosomal DNA from strains ISP479C and ISP479R and two tPMP^r transductants (A and B) of strain ISP479C (transduced from strain ISP479R). The probe is Tn917 (shares >90% homology with Tn551). A *sar* mutant resulting from a single Tn917 insertion serves as a positive control; an internal *Eco*RI site within the *sar* locus leads to two hybridizing bands with the probe.

a 2-h exposure in tPMP). Southern blot analysis of chromosomal DNA isolated from the tPMP-resistant transductant colonies confirmed a single insertion of Tn551, with a hybridization pattern similar to that seen within strain ISP479R. This finding strongly suggested that a single Tn551 had inserted within a chromosomal location in the transductants, identical to that of the tPMP^r donor strain, ISP479R (Fig. 3).

Valvular adherence and bacteremia clearance. No significant differences in either the rates of early bacteremia clearance (1 and 30 min after i.v. inoculation) or early bacterial adherence to sterile vegetations (30 min after i.v. inoculation) were observed in catheterized rabbits challenged with either strain ISP479C or ISP479R (Table 2).

Induction of endocarditis by tPMP^s and tPMP^r strains. The frequencies of endocarditis induction were similar in catheterized rabbits challenged with strains ISP479C or ISP479R, across the i.v. challenge inoculum range of 10^3 to 10^6 CFU (Table 3).

Intravegetation staphylococcal densities achieved by the

TABLE 2. Clearance of bacteremia and valvular adherence of tPMP^s and tPMP^r *S. aureus* strains

Inoculum ^a and strain	Blood culture (mean log ₁₀ CFU/ml $\pm$ SD) after		Valvular culture (mean CFU/vegetation $\pm$ SD)
	1 min	30 min	
10^6 CFU			
ISP479C	4.2 $\pm$ 0.6	1.2 $\pm$ 0.7	49 $\pm$ 23
ISP479R	3.9 $\pm$ 0.7	1.3 $\pm$ 0.5	92 $\pm$ 43
10^7 CFU			
ISP479C	4.6 $\pm$ 1.5	1.68 $\pm$ 0.23	395 $\pm$ 22
ISP479R	4.4 $\pm$ 0.2	1.51 $\pm$ 1.4	324 $\pm$ 89

^a At inocula lower than 10^6 CFU, there was no detectable adherence of bacterial colonies on sterile vegetations. Each challenge inoculum for each strain was administered i.v. to six animals with indwelling transaortic catheters.

TABLE 3. Endocarditis induction rates in catheterized rabbits

Inoculum challenge (CFU)	No. developing endocarditis/no. challenged (%) with strain:	
	ISP479C	ISP479R
10^3	6/14 (43)	5/15 (33)
10^4	6/9 (67)	11/17 (65)
10^5	11/11 (100)	10/10 (100)
10^6	9/9 (100)	10/10 (100)

tPMP^r strain at 24 h postinfection were substantially higher than those observed in animals challenged with the tPMP^s strain, across the entire i.v. challenge inoculum range (range, ~ 1 to $2 \log_{10}$ CFU/g differences) (Fig. 4). Moreover, when catheterized rabbits were challenged with a 100% infective dose inoculum of either staphylococcal strain, significant differences in achievable vegetation densities were observed on each daily sacrifice date (Table 4). Thus, final vegetation densities of ISP479R exceeded those of strain ISP479C by ~ 2 to $5 \log_{10}$ CFU/g over this 7-day postinfection period.

Retention of tPMP susceptibility phenotype in vivo. In animals infected with strain ISP479C and serially sacrificed over a 7-day postinfection period, no bacterial growth was observed on plates containing protamine at its MIC for this strain (0.5 mg/ml). Moreover, the direct testing of selected ISP479C colonies from protamine-free vegetation culture plates for tPMP susceptibility in vitro confirmed the retention of tPMP susceptibility for this strain in vivo (data not shown). Similarly, in animals challenged with strain ISP479R and serially sacrificed over a 7-day postinfection period, bacterial growth was observed on plates containing 0.5 mg of protamine per ml but not on plates containing 10 mg of protamine per ml (the protamine MIC for this strain). In addition, direct tPMP susceptibility testing of selected ISP479R colonies from protamine-free vegetation culture plates confirmed the in vivo retention of tPMP resistance.

Southern hybridization of vegetation colonies. Several colonies obtained from vegetation cultures of animals infected with strain ISP479R were processed for Southern blotting as above. These studies confirmed retention of the single Tn551 insert within the identical chromosomal locus of strain ISP479R following animal passage (data not shown).

DISCUSSION

Thrombin is readily generated in vivo at sites of endovascular damage via release of local procoagulant molecules, such as tissue factor, from endothelial cells or subendothelial stroma (8, 9). Of note, our laboratory (32) and others (7) have demonstrated that physiological levels of thrombin can, in turn, prompt the release from rabbit platelets of an endogenous antimicrobial peptide that we have termed tPMP (32). We have recently shown that tPMP has a number of direct microbiological effects in vitro against common endovascular pathogens (e.g., staphylococci and *Candida*), including microbial killing (29, 30, 32), postexposure growth inhibition (31), and anti-platelet adherence and aggregation (34).

The growth and maturation of infected cardiac vegetations in endocarditis are critically linked to the ability of the infecting pathogen to adhere to and aggregate platelets (26), as well as to resist local host defenses (2). Thus, the ability of tPMP to exert the above antimicrobial effects in vitro suggested that intrinsically tPMP^r microbial strains would have a selective survival advantage at sites of endovascular damage and would

FIG. 4. (a) Mean bacterial densities within culture-positive vegetations from catheterized rabbits challenged with strain ISP479C across a challenge inoculum range (10^3 to 10^6 CFU/ml). (b) Mean bacterial densities within culture-positive vegetations from catheterized rabbits challenged with strain ISP479R across a challenge inoculum range (10^3 to 10^6 CFU/ml).

possess an enhanced capacity to induce and propagate endocarditis in vivo. The present study was designed to precisely assess the influence of tPMP susceptibility on the pathogenesis of experimental staphylococcal endocarditis by using genetically related tPMP^s and tPMP^r *S. aureus* strains.

Platelets represent the predominant cell type found at sites of endocardial damage and also account for most of the weight of infected vegetations in endocarditis (27). Although tPMP has been shown to significantly impair the ability of *S. aureus* to adhere to platelets in vitro (34), we were unable to demonstrate a difference between the tPMP^s and tPMP^r *S. aureus* strains in terms of in vivo binding capacities to experimental valvular vegetations. Similarly, Dankert et al. (7) have recently shown that viridans streptococci which differ in intrinsic susceptibility to platelet microbicidal proteins adhere equally to experimental cardiac vegetations. These data suggested that the major influences of tPMP susceptibility on endocarditis pathogenesis relate to events subsequent to postvalvular adherence. This observation is not unexpected, since endovascular pathogens, such as *S. aureus*, possess a repertoire of surface adhesins that enable this organism to bind to important matrix proteins present at sites of damaged endocardium, particularly fibrinogen (12, 15, 19). This latter concept was supported in the present study by the comparable capacity of the tPMP^s and tPMP^r strains to bind fibrinogen in vitro. Furthermore, the ability of the tPMP^s and tPMP^r *S. aureus* strains to bind equally

to sterile endocardial vegetations in vivo correlated with an equivalent capacity of each strain to induce experimental endocarditis.

In the present study, the major in vivo difference between the tPMP^s and tPMP^r *S. aureus* strains in experimental endocarditis was in terms of achievable vegetation densities. Thus, over a 7-day postinfection period, intravegetation staphylococcal densities in animals challenged with the tPMP^r strain were significantly higher than in animals infected with the tPMP^s strain. These data parallel our prior studies in which a tPMP^s viridans streptococcus was used to induce experimental endocarditis in the presence or absence of selective thrombocytopenia. In thrombocytopenic animals with endocarditis, intravegetation streptococcal densities were significantly higher than in animals with normal platelet counts (27). In addition, we have recently demonstrated that a tPMP^r *Candida* strain reached significantly higher fungal densities within experimental cardiac vegetations than did a genetically related tPMP^s strain; moreover, only the tPMP^r strain was able to undergo hematogenous dissemination to the spleen (33). Collectively, these latter investigations, as well as our present data, strongly suggest that platelets play a critical role in limiting microbial proliferation within, and hematogenous dissemination from, infected vegetations, provided that the infecting organism is susceptible to the microbicidal effects of tPMP. In contrast, organisms which are intrinsically tPMP^r may be able to circum-

TABLE 4. Serial vegetation densities in animals infected with genetically related tPMP^s and tPMP^r *S. aureus*^a strains

Strain	Vegetation density (log ₁₀ CFU/g of vegetation ± SD) on:					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
ISP479C (tPMP ^s)	6.43 ± 2.20	6.42 ± 0.40	5.52 ± 1.02	6.96 ± 0.13	8.07 ± 1.15	7.03 ± 0.11 ^b
ISP479R (tPMP ^r)	8.28 ± 0.37	10.67 ± 0.4	10.4 ± 0.28	10.3 ± 0.25	10.4 ± 0.54	10.8 ± 0.40 ^b
P values	0.008	0.047	0.016	0.000024	0.033	0.0001

^a The challenge inoculum in all catheterized rabbits was $\sim 4 \times 10^7$ CFU i.v.

^b Initial in vitro tPMP susceptibilities retained among vegetation clones (tPMP^s clones, <20% survival at 2 h after tPMP exposure; tPMP^r clones, >90% survival at 2 h after tPMP exposure).

vent such mitigating effects of tPMP and undergo unbridled replication within cardiac vegetations.

We have previously shown that bacteremic staphylococcal strains isolated from patients with clinical evidence of endocarditis were significantly more resistant to tPMP in vitro than were bacteremic strains from patients without endocarditis (predominantly catheter sepsis [29]). This suggested one of three paradigms to explain the preponderance of tPMP^r staphylococci at endocardial infection sites: (i) staphylococci which cause endocarditis are intrinsically tPMP^r, which gives them a selective survival advantage at sites of endocardial damage; (ii) bacteremic staphylococci are heterotypically tPMP^r (like methicillin-resistant strains [14]), with a small tPMP^r subpopulation emerging at sites of endocardial damage following eradication of the major tPMP^s population by the local platelet release of tPMP; or (iii) staphylococcal strains are homogeneously tPMP^s at the time of initial colonization of damaged endocardium but develop a stable tPMP^r phenotype following repetitive exposure to sublethal tPMP concentrations at this site (similar to the manner in which stable tPMP^r *S. aureus* strains can be derived in vitro [31, 34]). The data from our present study would tend to discount this latter mechanism, since the tPMP^s phenotype of strain ISP479C was retained in vivo over a 7-day postinfection period in the experimental endocarditis model. Moreover, the in vitro population analysis in the present study would tend to negate the possibility that bacteremic strains of *S. aureus* are predominantly heterotypic. However, to completely evaluate this latter possibility, we are currently performing detailed population analyses of well-characterized bacteremic staphylococcal strains from patients with or without clinical evidence of endocarditis to answer this important question (29). Lastly, a single transposon insert was documented within the chromosomal DNA of the tPMP^r strain (ISP479R). This transposon was transduced back into the original tPMP^s parental strain (ISP479C), with conversion of the transductant to a tPMP^r phenotype in vitro. The presence of these isogenic strain pairs differing in tPMP susceptibility now provides a powerful tool to examine the genetic basis of tPMP^r in *S. aureus*. Until this is carried out, the precise relationship between intrinsic tPMP^r in wild-type staphylococci and that exhibited by the transposon mutant strain in the current study remains undefined. Moreover, the physiologic basis on which the genetic interruption in the tPMP^r strain confers tPMP resistance remains to be elucidated. Preliminary lipid analyses of our isogenic strain pair indicate that the tPMP^r mutant possesses a substantially more fluid cytoplasmic membrane than does the parental, tPMP^s strain (3a). This altered phenotype may well influence the interaction of tPMP with the cytoplasmic membrane, a principal target for tPMP-induced staphylocidal effects (36).

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