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Resistance to Platelet Microbicidal Protein Results in Increased Severity of Experimental *Candida albicans* Endocarditis

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Thrombin-induced platelet microbicidal protein (tPMP) exerts potent in vitro microbicidal activity against pathogens commonly found in the bloodstream, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Candida albicans*. Localized platelet release of tPMP may be important in defense against infections involving the vascular endothelium caused by tPMP-susceptible organisms. In contrast, pathogens capable of surviving in the presence of tPMP could then exploit the platelet as an adhesive surface for attachment to damaged endothelium. To examine these hypotheses, we derived a tPMP-resistant ($tPMP^r$) *C. albicans* strain from its tPMP-sensitive ($tPMP^s$) parental strain ATCC 36082 by serial passage in tPMP (5 μg/ml). The $tPMP^r$ and $tPMP^s$ strains were equivalent in vitro as assessed by genotyping (electrophoretic karyotype and restriction endonuclease analysis of genomic DNA), biotyping, germination, platelet aggregation, adherence to vascular endothelial cells, and growth characteristics. In addition, the $tPMP^r$ phenotype was stable following multiple in vitro and in vivo passages. We then investigated the in vivo relevance of tPMP susceptibility on endovascular infection using a rabbit model of endocarditis and hematogenous dissemination. Rabbits with transaortic catheters ($n = 15$ in each group) were challenged with either the $tPMP^s$ or $tPMP^r$ *C. albicans* strain. All rabbits developed *C. albicans*-induced endocarditis, as determined by the presence of infected vegetations. In rabbits challenged with the $tPMP^r$ *C. albicans* strain, significantly higher mean vegetation fungal densities occurred than in animals challenged with the $tPMP^s$ strain ($P < 0.001$). These results were seen in the absence of differences in either initial adherence of strains to cardiac valves or vegetation weights. Furthermore, although these *C. albicans* strains induced equivalent rates and extent of hematogenous renal infection, only the $tPMP^r$ strain disseminated hematogenously to the spleen (15 of 15 rabbits) versus 0 of 15 [$tPMP^s$ strain]; $P < 0.0001$). Thus, $tPMP^r$ *C. albicans* caused more-severe endocarditis and produced greater metastatic sequelae than the $tPMP^s$ counterpart.

Fungemia due to *Candida* species occurs at a frequency of 0.5 to 2.0 per 10,000 hospital admissions, and these organisms now represent 5 to 10% of all nosocomial bloodstream isolates in the United States (10, 27, 36). As a result, *Candida albicans* is the most common fungal pathogen causing endovascular infections, such as infective endocarditis, vascular catheter sepsis, and infections of vascular prostheses (11, 28, 36). Candidemia and hematogenously disseminated candidiasis are increasing in incidence and are particularly prevalent in neutropenic and immunocompromised patients and in postoperative patients receiving long-term intravenous antibiotic therapy or hyperalimentation (13, 23, 26, 31, 32). Moreover, hematogenously disseminated candidiasis is a serious and often life-threatening infection, with an attributable mortality rate of 38 to 50% (10, 11).

The mechanisms by which *C. albicans* initiates endovascular infection and causes hematogenous dissemination are believed to be complex, involving the interaction of the blood-borne organism with specific host plasma proteins (fibrinogen and fibronectin, etc.), endothelial cells, and platelets (3, 4, 15–21, 29). Furthermore, *C. albicans* must avoid complement- and cell-mediated (neutrophil and macrophage) killing mechanisms. The traditional view of platelet function in candidal pathogenesis has been to promote seeding of the vascular endothelium by providing focal sites for candidal adhesion (4, 9, 15, 20, 30). Several studies have demonstrated that *C. albicans* adheres to platelet-fibrin matrices in vitro and in vivo during the induction of experimental infective endocarditis (3, 4, 20, 21). Moreover, Klotz et al. reported that aggregated platelets enhance the adherence of *C. albicans* to endothelial cells in vitro (16). Following adhesion, *C. albicans* must subsequently traverse the vascular endothelium to cause infection of the tissue parenchyma (12, 15). In addition, endovascular foci of infection affords *Candida* organisms an ongoing opportunity for hematogenous dissemination and metastatic seeding of distant organs.

In contrast, recent evidence from our laboratory suggests that platelets may act to limit the induction and/or progression of endovascular infection. In vitro studies have shown that thrombin can prompt the release of a low-molecular-mass cationic antimicrobial peptide (39, 42). This peptide, termed thrombin-induced platelet microbicidal protein (tPMP) (42), has been shown to exert potent in vitro fungicidal activity against *Candida* species as well as *Cryptococcus neoformans* (39). In addition, tPMP reduces *C. albicans* adherence to platelets in vitro (43). Although yet to be confirmed, it is intuitively likely that physiological levels of thrombin, similar to those gener-

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ated at sites of endovascular damage (6–8), cause platelets to release tPMP in vivo. Thus, our hypothesis contends that the in vivo release of tPMP may limit *C. albicans* infection at sites of endothelial damage. Our current study was designed to examine the influence of in vitro tPMP susceptibility of *C. albicans* on the induction and progression of endocarditis in vivo in a well-characterized animal model.

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MATERIALS AND METHODS

tPMP. tPMP was prepared as previously described (39, 42). In brief, blood from New Zealand White rabbits was collected into siliconized tubes containing a citrate anticoagulant. Anticoagulated whole blood was then centrifuged (75 × *g*, 10 min) to produce a platelet-rich plasma supernatant containing <1% leukocyte contamination. Platelets were pelleted by centrifugation (1,000 × *g*, 10 min) of the upper two-thirds of the platelet-rich plasma 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 (MEM; 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 (1 U/ml, 37°C, 20 min; Sigma Chemical Co.) in the presence of 0.2 M $CaCl_2$. Following thrombin-induced platelet activation, residual platelet material was removed by centrifugation, and the tPMP-rich supernatant was recovered. Substantial purification (approximately 25-fold, ≥80% purity) of tPMP was achieved by anionic (cellulose acetate/nitrate) affinity adsorption and cationic elution as previously described (39, 42). Preparations rich in tPMP were then pooled, dialyzed against 10 volumes of sterile deionized water (molecular mass cutoff of 3.5 kDa, Spectrapor 3; Spectrum Medical Industries, Los Angeles, Calif.), and stored at −20°C.

Bioactivity of tPMP preparations. The bioactivity of the tPMP preparations was determined by previously described methods (39, 42). In brief, bioassays were performed with *Bacillus subtilis* (ATCC 6633), an indicator organism highly sensitive to the bactericidal action of tPMP (42). For the bioassay, *B. subtilis* was grown in brain heart infusion 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 [NS]), and resuspended in phosphate-buffered saline (PBS; 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 μl). After 30 min of incubation at 37°C, a 20-μl aliquot was removed from each well, diluted into PBS containing 0.01% (wt/vol) sodium polyanethol sulfonate (Sigma Chemical Co.) to inhibit further PMP-induced bacterial killing, and quantitatively cultured on 6.6% sheep blood agar. tPMP bioactivity (units per milliliter) was defined as the inverse of the highest tPMP dilution which retained ≥95% lethality versus *B. subtilis* within the 30-min assay time period (42). The specific activity of each tPMP preparation was approximately 50 U/μg of protein as determined by spectrophotometry (λ, 220 nm).

***Candida* strains.** All fungal isolates were stored on Sabouraud dextrose agar slants at 4°C. Organisms were routinely cultured for 16 to 18 h in yeast-nitrogen base (YNB) broth (Difco Laboratories) enriched with 0.5% glucose (wt/vol) at 27°C on a rotating drum as previously described (12). After being harvested by centrifugation (3,000 × *g*, 10 min), the yeast cells were resuspended in NS, sonicated for 4 s to ensure singlet organisms (60 Hz, sonifier model 350; Branson, Danbury, Conn.), washed twice in NS, and sonicated again. The cells were then counted in a hemacytometer and adjusted to the desired concentration in PBS. Yeast concentrations determined by hemacytometry were confirmed by quantitative culture.

C. albicans ATCC 36082 is a well-characterized clinical isolate that consistently induces experimental infective endocarditis (37). Its susceptibility to tPMP was determined by exposing 10^6 blastospores to 5 μg of tPMP per ml for 2 h at 37°C. We derived a tPMP-resistant (tPMP^r) *C. albicans* strain from the tPMP-sensitive (tPMP^s) parental strain (10^6 blastospores) by serial passage of a single isolated colony in 5 μg of tPMP per ml of MEM for 3 h at 37°C. Following each tPMP exposure, organisms were quantitatively cultured on YNB agar, and the percent survival of the inoculum was calculated. A surviving colony was selected, cultured in YNB medium, and reexposed to tPMP as described above. After eight in vitro passages in 5 μg of tPMP per ml, a *C. albicans* strain exhibiting a significantly reduced susceptibility to tPMP was isolated.

Phenotypic comparison of the tPMP^s and tPMP^r *C. albicans* strains. The ability of the tPMP^s and tPMP^r *C. albicans* strains to assimilate a panel of 19 carbohydrates was compared by using the API20c clinical yeast system (BioMérieux Vitek, Inc., Hazelwood, Mo.) following manufacturer's instructions. In addition, the tPMP^s and tPMP^r strains were assessed for similarities in their in vitro growth kinetics in the presence and absence of protamine sulfate (0, 3, or 7 mg/ml; Sigma), in germination rates after incubation in pooled healthy human serum for 2 h at 37°C, and in colonial morphology by visual inspection after 24 h of growth on YNB agar. The tPMP^s and tPMP^r *C. albicans* strains were also compared for their in vitro adherence to human umbilical vein endothelial cells in vitro as previously described (25). For these assays, the adherence of each *C. albicans* strain to endothelial cell monolayers in six-well tissue culture plates was determined following 30 min of incubation. After rinsing to remove nonadherent organisms, endothelial cell monolayers were overlayed with 2 ml of Sabouraud dextrose agar. Colonies representing adherent organisms were counted after 24 h of incubation at 37°C, and the results were expressed as the percent adherence of the initial inoculum. In addition, the stability of the tPMP^r phenotype was compared before and after eight in vitro passages in tPMP-free YNB medium and following three in vivo passages in rabbits, by determining the percent survival of each strain in 5 μg of tPMP per ml of MEM for 2 h as described above. To compare the abilities of the tPMP^s and tPMP^r strains to aggregate rabbit platelets in vitro, platelet aggregometry was also performed (as previously described [41]), with a *Candida* blastospore-to-platelet ratio of 10:1.

Genotypic comparison of tPMP^s and tPMP^r *C. albicans* strains. The genotypic profiles of the parental (tPMP^s) and derived (tPMP^r) *C. albicans* strains were compared. Molecular typing of each isolate was performed by Michael Pfaller and Richard Hollis, University of Iowa College of Medicine, as previously described (2, 35). The tPMP^s and tPMP^r strains were compared by two methods: (i) electrophoretic karyotype analysis with a CHEF-DR II (Bio-Rad, Hercules, Calif.) and (ii) restriction endonuclease analysis of genomic DNA with the restriction enzymes *Bss*HII and *Sfi*I (Biolabs, Beverly, Mass.). Analyses of electrophoretic karyotype and restriction endonuclease genomic digestion profiles were performed by visual inspection of ethidium bromide-stained agarose gels. tPMP^s and tPMP^r *C. albicans* isolates were considered to be genotypically different when any readily detectable DNA bands did not migrate identically. Lambda phage concatemeric DNA (FMC, Rockland, Maine) was routinely used for molecular size comparison. DNA from *Saccharomyces cerevisiae* was used as a control in karyotyping analyses.

Rabbit model of *C. albicans*-infective endocarditis. The rabbit model of experimental infective endocarditis was used in these studies (37). Female New Zealand White rabbits (weight, approximately 2.5 kg each) were caged separately and provided food and water ad libitum. The animals were anesthetized by intramuscular injection of 35 mg of ketamine hydrochloride (Ketaset; Aveco Co., Ft. Dodge, Iowa) per kg of body weight and 1.5 mg of xylazine (Rompun; Mobay Co., Shawnee, Kans.) per kg. Sterile endocardial vegetations were produced by transaortic valvular placement of a sterile polyethylene catheter (internal diameter, 0.86 mm; Beckton Dickinson and Co., Parsippany, N.J.) as previously described (37). The catheter remained in place for the duration of the experiment. Infective endocarditis was produced 48 h after catheterization by intravenous injection of 2 × 10^7 CFU of either the tPMP^s or tPMP^r *C. albicans* strain via the marginal ear vein. Pilot studies were performed to confirm that this challenge inoculum resulted in infective endocarditis in >95% of the catheterized animals for each strain. To generate adequate statistical power, 15 rabbits each were catheterized and challenged with either the tPMP^s or tPMP^r *C. albicans* strain.

Comparison of early stages of endocarditis due to tPMP^s and tPMP^r *Candida* strains. In vivo adherence studies were performed to compare the abilities of the two *C. albicans* strains to initially adhere to cardiac valves in the animal model described above. For these studies, two groups of five animals each were catheterized and then challenged 48 h after catheterization by intravenous injection of 2 × 10^7 CFU of either the tPMP^s or tPMP^r *C. albicans* strain via the marginal ear vein as described above. To assess and quantitatively compare the clearance of fungemia of the two strains following intravenous challenge, blood samples were obtained 1, 5, and 30 min postchallenge and quantitatively cultured on YNB agar. The animals were sacrificed at 30 min postchallenge as described above, and the cardiac valves were removed, homogenized, and quantitatively cultured as previously described (37). Following incubation, colonies were enumerated, and the mean number of CFU (± standard deviation) per gram of cardiac valve was calculated for each strain and expressed as the normalized percentage of the initial inoculum adhering to the traumatized cardiac valve (29).

Induction of *C. albicans* endocarditis and hematogenous dissemination. The animals were sacrificed 72 h after intravenous challenge with either the tPMP^s or tPMP^r *C. albicans* strain. Rabbits were euthanized with 200 mg of sodium pentobarbital (Abbott Laboratories, Chicago, Ill.) administered by rapid intravenous injection. At the time of sacrifice, the heart, kidneys, and spleen of each animal were aseptically removed. Proper catheter position was verified, and cardiac vegetations of each animal were removed, pooled, weighed, and homogenized in sterile NS. Vegetation homogenates were then serially diluted, quantitatively cultured in parallel on YNB agar with or without 5 mg of protamine sulfate per ml to screen for tPMP susceptibility (see below). Following incubation at 37°C for 48 h, colonies were enumerated, and the mean number of CFU per g of tissue (± standard deviation) was determined. Induction of endocarditis was microbiologically confirmed by culture-positive vegetations. Colonies growing on protamine-containing YNB medium were subsequently assayed to quantify their level of tPMP resistance as described above. In parallel, the spleen and kidneys were macroscopically examined for abscesses, and each organ was then homogenized and quantitatively cultured as described above. The mean number of CFU/g kidney or spleen tissue (± standard deviation) was then calculated.

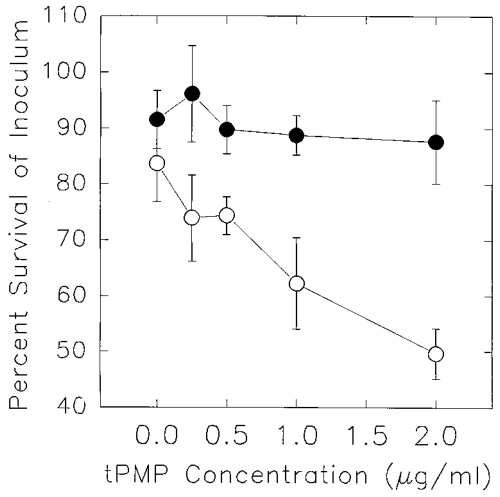

FIG. 1. Population analyses of tPMP^s and tPMP^r *C. albicans* strains. Each strain was resuspended to an inoculum of 10^3 CFU/ml and exposed to tPMP at a range of concentrations at 37°C for 3 h. Following exposure of the strains to each tPMP concentration, aliquots were removed and quantitatively cultured on YNB agar. Colonies were then enumerated, and the percent survival of the initial inoculum was calculated. Results represent the means of three independent experiments performed in duplicate. ○, tPMP^s *C. albicans*; ●, tPMP^r *C. albicans*.

Qualitative screen for tPMP^r *C. albicans*. The bioactivity of tPMP is significantly reduced in solid media, such as YNB agar, preventing use of tPMP-containing agar in quantitative culturing assays (unpublished data). Our pilot studies demonstrated that the in vitro susceptibilities of the tPMP^s and tPMP^r *C. albicans* strains to the cationic antimicrobial protein protamine sulfate mirrored those of tPMP. Thus, although both strains grew normally in agar containing 3 mg of protamine per ml, only the tPMP^r strain grew normally in YNB medium containing 7 mg of protamine sulfate (Sigma Chemical Co.) per ml, while the tPMP^s strain failed to grow under these conditions. Therefore, protamine sulfate was employed as a surrogate agent for tPMP when screening for the tPMP^r phenotype of *C. albicans* cells cultured from tissues of experimental animals. Organisms which grew on YNB agar containing 7 mg of protamine per ml were then analyzed for tPMP susceptibility by the microbicidal assay described above.

Statistical analyses. Proportional differences in the frequencies of endocarditis induction and hematogenous seeding to kidneys or spleen were compared by the Fisher's exact test. Differences in fungal densities within cardiac vegetations, kidneys, and spleen were compared by the Wilcoxon rank sum test. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Susceptibility of *C. albicans* to tPMP. *C. albicans* ATCC 36082 exhibited a mean survival of 31.3% ± 3.6% of the 10^6 CFU/ml blastospore inoculum after 2 h of exposure to 5 μg of tPMP/ml. On the basis of previous studies indicating a 50% survival breakpoint for tPMP susceptibility versus resistance (5, 38), this strain was considered tPMP susceptible (tPMP^s). Following eight in vitro serial exposures to tPMP, we recovered a *C. albicans* strain exhibiting a mean survival of 72.8% ± 8.2% of the 10^6 CFU/ml blastospore inoculum. On the basis of the same breakpoint criteria described above, this strain was considered to have increased resistance to tPMP (tPMP^r).

Stability of *C. albicans* tPMP^s and tPMP^r strains. The in vitro and in vivo stabilities of the tPMP susceptibility phenotypes were determined for each *C. albicans* strain after eight passages in YNB medium and three passages through the rabbit endocarditis model. The tPMP^s and tPMP^r *C. albicans* strains retained their respective prepassage tPMP susceptibility profiles, indicating that these phenotypes were stable. Similarly, the susceptibility profiles of these strains to protamine sulfate (as a surrogate for tPMP susceptibility) were also retained following in vitro and in vivo passages. In addition, we assessed the tPMP susceptibilities of populations of cells originating from a single colony of either the tPMP^s or tPMP^r strain. We found that the two strains maintained their respective tPMP susceptibility phenotypes for a wide range of tPMP concentrations (for instance, mean survival of 49.6% ± 8.2% or 88.1% ± 12.8%, respectively [$n = 3$ each], when exposed to 2 μg of tPMP per ml) (Fig. 1). Therefore, these results demonstrated that the tPMP^s and tPMP^r strains were distinct, homogeneous populations with respect to their different tPMP susceptibilities.

Phenotypic and genotypic comparisons of *C. albicans* tPMP^s and tPMP^r strains. The *C. albicans* tPMP^s and tPMP^r strains were found to be indistinguishable in colonial morphology (white, mucoid) and germination rate (data not shown). Additionally, they caused similar profiles of rabbit platelet aggregation (i.e., equivalent rate and extent of platelet aggregation; data not shown). The in vitro growth kinetics of the two strains in YNB broth alone and YNB broth containing a sublethal concentration of protamine sulfate (3 mg/ml) were not different (Fig. 2); however, significant differences were exhibited in growth kinetics in the presence of 7 mg of protamine per ml, as expected (Fig. 2). Furthermore, the tPMP^s and tPMP^r strains were not significantly different in adherence to human umbilical vein endothelial cells in vitro (mean adherence [$n = 3$] of 44.0% ± 10.7% and 48.9% ± 7.5%, respectively). In addition, biotyping of these strains revealed no differences in their abilities to assimilate 19 different carbohydrates (API biotyping). Electrophoretic karyotype analysis revealed no detectable differences in the genotypic profiles of the tPMP^s and tPMP^r *C. albicans* strains (Fig. 3A). Moreover, restriction endonuclease analyses using *Bss*HII or *Sfi*I revealed no detectable genotypic differences between these two strains (Fig. 3B and C). Collectively, these findings indicate that the tPMP^s and tPMP^r strains of *C. albicans* were phenotypically and genotypically equivalent as assessed by these methods.

Early adherence and fungemia due to tPMP^s and tPMP^r *C.*

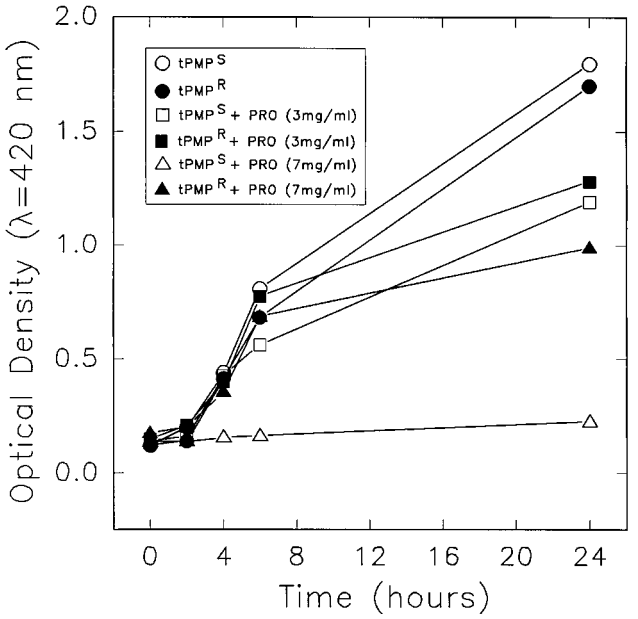

FIG. 2. Comparison of growth kinetics of tPMP^s and tPMP^r *C. albicans* strains in YNB medium with and without protamine sulfate (PRO). The initial inoculum was 10^5 CFU/ml; all cultures were incubated at 37°C. Only when protamine was increased to 7 mg/ml (to discriminate tPMP^s and tPMP^r strains) was there a significant difference in growth kinetics of the two strains.

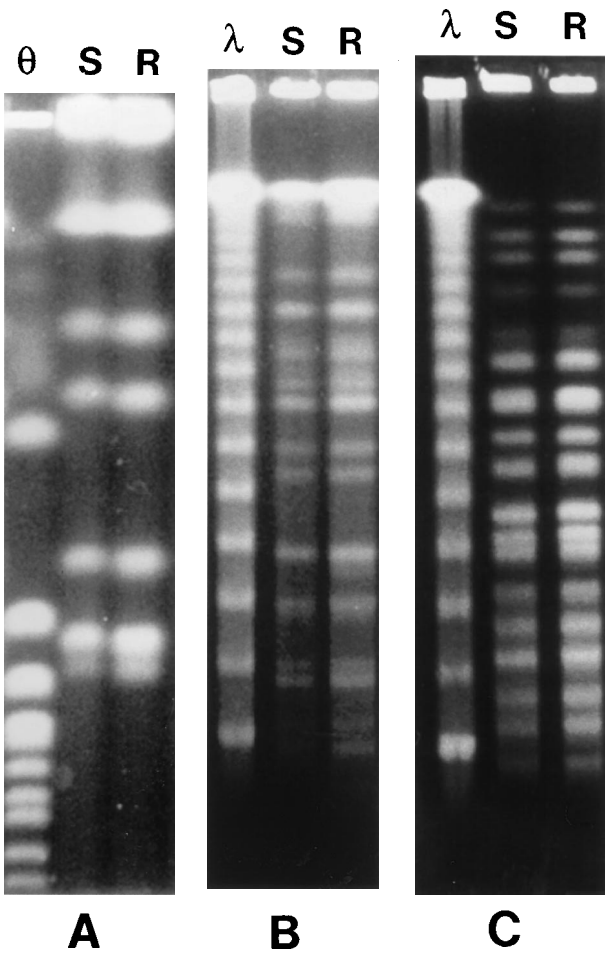

FIG. 3. Genotypic comparison of *C. albicans* tPMP^s and tPMP^r strains. (A) Karyotype analysis of *C. albicans* genomic DNA by pulsed-field gel electrophoresis (2, 35). Lanes: Θ, *Saccharomyces cerevisiae* chromosomal DNA as molecular size standards; S, *C. albicans* tPMP^s strain; R, *C. albicans* tPMP^r strain. Restriction endonuclease digestion analysis of *C. albicans* genomic DNA with *Sfi*I (B) and *Bss*HII (C) followed by pulsed-field gel electrophoresis (2, 35). Lanes: λ, lambda phage DNA concatamers as molecular size standards; S, *C. albicans* tPMP^s strain; R, *C. albicans* tPMP^r strain.

***albicans* strains.** As summarized in Table 1, no significant differences were observed in the levels of fungemia at 1, 5, or 30 min postchallenge. In addition, there were no significant differences detected in the abilities of these strains to adhere to damaged (catheterized) cardiac valve endothelium (Table 1). Thus, the development of tPMP resistance in the tPMP^r strain did not influence its valvular adherence or the rate at which it was cleared from the circulation compared with that in the tPMP^s strain.

TABLE 1. Comparison of tPMP^s and tPMP^r *C. albicans* strains in early stages of infective endocarditis

Characteristic	Result for *C. albicans* strain[a]	
	tPMP^s	tPMP^r
Early valve adherence (%)[b]	4.04 ± 1.98	4.84 ± 1.59
Fungemia (CFU/ml) at:		
1 min	3.12 ± 0.41	2.95 ± 0.49
5 min	1.83 ± 0.24	2.21 ± 0.38
30 min	1.23 ± 0.51	1.64 ± 0.55

[a] Data represent mean values (± standard deviations) obtained from two groups containing five animals each. There were no significant differences between any of the parameters compared ($P \geq 0.05$).

[b] Results are expressed as the normalized percentage of the initial inoculum adhering to the traumatized cardiac valve (adherence ratio) (28) ± standard deviations at the times postchallenge indicated.

TABLE 2. Influence of *C. albicans* tPMP susceptibility in vitro on endocarditis and hematogenous dissemination in vivo

Outcome	Result for *C. albicans* strain[a]	
	tPMP^s	tPMP^r
Endocarditis induction[b]	15/15	15/15
Vegetation fungal density[c]	5.29 ± 0.14	6.37 ± 0.11*
Dissemination to kidney[b]	15/15	15/15
Kidney fungal density[c]	3.66 ± 0.21	3.64 ± 0.18
Dissemination to spleen[b]	0/15	15/15**
Splenic fungal density[c]	<0.1	2.89 ± 0.16**
C. albicans phenotype ex vivo[d]		
Vegetation	Pro^s tPMP^s	Pro^r tPMP^r
Kidney	Pro^s tPMP^s	Pro^r tPMP^r
Spleen	NA (sterile)[e]	Pro^r tPMP^r

[a] *, $P \leq 0.001$ versus tPMP^s *C. albicans*; ** $P \leq 0.00001$ versus tPMP^s *C. albicans*.

[b] Results expressed as number of animals positive per number of animals tested.

[c] Results expressed as mean CFU per gram of tissue ± standard deviation.

[d] Pro, protamine sulfate (surrogate marker for tPMP susceptibility); tPMP, 5 μg/ml and 2 h of exposure.

[e] NA, not applicable.

Induction of endocarditis by tPMP^s and tPMP^r *C. albicans* strains. Both the tPMP^s and tPMP^r *C. albicans* strains induced infective endocarditis in all rabbits challenged (15 of 15 rabbits for each strain) (Table 2). Additionally, there were no significant differences in vegetation weights in the animals challenged with either the tPMP^s or tPMP^r strain (means of 0.1144 ± 0.064 and 0.1198 ± 0.093 g, respectively [$n = 5$ for each group]). However, the tPMP^r strain achieved significantly higher mean fungal vegetation densities than did the tPMP^s parental strain ($P < 0.001$; Table 2). Organisms recovered from cardiac vegetations of animals infected with the tPMP^s strain retained the tPMP^s phenotype and failed to grow in the presence of protamine (7 mg/ml). Similarly, organisms isolated from vegetations of animals challenged with the tPMP^r strain grew normally in YNB medium containing protamine and retained the tPMP^r phenotype in tPMP assays (Table 2).

Hematogenous dissemination of tPMP^s and tPMP^r *C. albicans* strains. In contrast, there was a significant difference in the abilities of the tPMP^s and tPMP^r *C. albicans* strains to disseminate to the spleen. No animals with endocarditis caused by the tPMP^s strain exhibited splenic abscesses, while all 15 animals with endocarditis caused by the tPMP^r *C. albicans* strain had macroscopic splenic abscesses, which were confirmed by quantitative culture ($P < 0.0001$; Table 2). Furthermore, *C. albicans* organisms recovered from infected spleens were consistently tPMP^r as assessed by growth in the presence of protamine and by subsequent tPMP susceptibility assays. The tPMP^s and tPMP^r strains uniformly infected the kidneys of all animals (15 of 15 rabbits for each strain; Table 2). Furthermore, the tPMP^s and tPMP^r strains achieved equivalent kidney fungal densities ($P > 0.05$; Table 2). In addition, *C. albicans* isolated from infected kidneys retained the original tPMP susceptibility phenotype (either tPMP^s or tPMP^r).

DISCUSSION

There is a growing body of evidence to support the hypothesis that platelets function to limit the induction and progression of endovascular infections. In vitro evidence indicates that *C. albicans* causes endothelial cell damage (12). Tissue factor, released from damaged or infected vascular endothelium (1, 6–8), subsequently generates thrombin from prothrombin at these sites (1, 6, 8). We have previously shown that rabbit platelets release tPMP when stimulated with physiological concentrations of thrombin in vitro (42). Thus, it is highly likely that candidal damage or colonization of vascular endothelial cells results in thrombin generation and subsequent localized release of tPMP from platelets in vivo.

Several recent in vivo investigations support the concept that platelets act to defend against endovascular infection through the release of tPMP. In collaboration with Sullam et al., our laboratory demonstrated that catheterized rabbits made selectively thrombocytopenic by an antiplatelet antibody exhibited significantly higher bacterial densities in cardiac vegetations than did healthy animals when infected with a $tPMP^s$ viridans-group streptococcus (34). In addition, Dankert et al. have shown that an anti-tPMP antibody administered to catheterized rabbits rendered them more susceptible to infective endocarditis than untreated control rabbits when challenged with a $tPMP^s$ streptococcal strain (5).

Our laboratory has shown that tPMP exerts in vitro fungicidal activity versus *C. albicans* (39). Furthermore, platelets bind *C. albicans* directly (24, 33, 43), suggesting a mechanism by which tPMP may be locally released from platelets in immediate proximity to these organisms. Recent findings also indicate that sublethal concentrations of tPMP reduce *C. albicans* adherence to, germination and damage of vascular endothelial cells in vitro (40). These observations suggest that tPMP may limit the induction, progression, and/or hematogenous dissemination of endovascular infections due to *C. albicans* by killing or the organism or reducing its interaction with endothelial cells at sites within the intravascular compartment. Collectively, these findings support the hypothesis that tPMP may modulate the principal pathogenic steps believed to be involved in *C. albicans* induction of infective endocarditis and hematogenous dissemination: (i) adherence to vascular endothelium, (ii) fungal germination, (iii) fungal proliferation, and (iv) fungal-induced damage of the vascular endothelium (11, 12, 15).

In the present study, we were able to induce significant tPMP resistance in *C. albicans* without inducing other detectable phenotypic or genotypic alterations. These respective $tPMP^s$ and $tPMP^r$ strains were found to represent relatively homogenous populations in their susceptibilities to tPMP. Although recent findings suggest that one or more *C. albicans* proteinases may protect the organism from humoral defense components in serum (14), the mechanism(s) of *C. albicans* resistance to tPMP remains undefined at present. Additionally, the $tPMP^s$ and $tPMP^r$ strains did not differ in the other phenotypic profiles (growth kinetics in the presence and absence of 3 mg of protamine per ml, platelet aggregation, adherence to vascular endothelial cells in vitro, adherence to cardiac valves in vivo, and metabolic biotyping) or genotypic profiles (electrophoretic karyotype and restriction endonuclease analysis) assessed in this study.

We used the $tPMP^s$ and $tPMP^r$ *C. albicans* strain pair to examine the influence of in vitro tPMP susceptibility on the ability of *C. albicans* to induce endocarditis and subsequently disseminate hematogenously in vivo. The $tPMP^r$ *C. albicans* strain achieved significantly greater fungal densities in cardiac vegetations than its $tPMP^s$ parental strain, despite equivalent rates of endocarditis induction. Moreover, although the $tPMP^s$ and $tPMP^r$ strains disseminated equally to the kidneys, only the $tPMP^r$ strain disseminated to the spleens in these animals. These results were observed in the absence of any significant differences between the $tPMP^s$ and $tPMP^r$ strains tested in early adherence to traumatized cardiac valves, development of valvular vegetations (no difference in vegetation weights), or clearance of fungemia over a 30-min period postchallenge.

These collective findings support several important conclusions regarding platelet and tPMP function in host defense against *C. albicans* endovascular infection in vivo. First, they indicate that the $tPMP^r$ phenotype may provide *Candida* organisms with a greater extent of proliferation at sites of endothelial damage and an enhanced ability to hematogenously disseminate to the spleen. This concept is supported by our previous data demonstrating that bacteremic *Staphylococcus aureus* isolates from patients with clinically confirmed endocarditis were significantly more resistant to tPMP in vitro than isolates from patients with *S. aureus* bacteremia in the absence of endocarditis (38). Second, our current data suggest that tPMP contributes to host defense by limiting *C. albicans* proliferation in endocarditis vegetations due to $tPMP^s$ strains. This antimicrobial function of tPMP may also enhance host defense against hematogenous seeding and abscess formation in the spleen caused by such organisms. This hypothesis is supported by the observations that the two strains tested were not significantly different in adherence to the vascular endothelium in vitro (vascular endothelial cells) or in vivo (cardiac valves) and were identical in their ability to aggregate platelets and produce valvular vegetations. Finally, our current findings also suggest that the importance of tPMP in host defense may differ in different vascular beds. For instance, our data indicate that tPMP may play a more important role in protecting the cardiac valve and splenic endothelium from microbial invasion than the renal endothelium. In this regard, microenvironmental conditions may influence the antimicrobial properties of tPMP in different tissues (e.g., differences in pH and osmolality in the kidneys versus the spleen). Additionally, as opposed to valvular and splenic infections, adhesion of microorganisms to platelets may not play a significant role in the induction and proliferation of renal infection. These possibilities will be the subject of future investigations.

In summary, our current findings support the hypothesis that platelets contribute to host defense against the induction and progression of infections involving the vascular endothelium. This potential host defense function of platelets may be effected by tPMP release from platelets in response to thrombin generated at sites of damage to vascular endothelial cells. Therefore, tPMP release may represent a specific response of the platelet to limit the establishment or evolution of infection involving the vascular endothelium.

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