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Thrombin-Induced Rabbit Platelet Microbicidal Protein Is Fungicidal In Vitro

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Platelet microbicidal protein (PMP) is released from platelets in response to thrombin stimulation. PMP is known to possess in vitro bactericidal activity against *Staphylococcus aureus* and viridans group streptococci. To determine whether PMP is active against other intravascular pathogens, we evaluated its potential fungicidal activity against strains of *Candida* species and *Cryptococcus neoformans*. Anionic resin adsorption and gel electrophoresis confirmed that the fungicidal activity of PMP resided in a small (~8.5-kDa), cationic protein, identical to previous studies of PMP-induced bacterial killing (M. R. Yeaman, S. M. Puentes, D. C. Norman, and A. S. Bayer, Infect. Immun. 60:1202–1209, 1992). When assayed over a 180-min period in vitro, the susceptibilities of these fungi to PMP varied considerably. Generally, *Candida albicans* strains (mean survival, 33.5% ± 6.9% [n = 6]) as well as isolates of *Candida glabrata* (mean survival, 50.8% ± 2.9% [n = 2]) were the most susceptible to killing by PMP, while *Candida guillermondii* and *Candida parapsilosis* were relatively resistant to PMP-induced killing. Compared with *C. albicans*, *C. neoformans* was relatively resistant to the fungicidal activity of PMP, with a mean survival among the isolates studied of 77.4% ± 12.4% (n = 6). Against *C. albicans*, PMP-induced fungicidal activity was time dependent (range, 0 to 180 min), PMP concentration dependent (range, 10 to 150 U/ml), and inversely related to the fungal inoculum (range, 5×10^3 to 1×10^5 CFU/ml). Scanning electron microscopy of PMP-exposed *C. albicans* and *C. neoformans* cells revealed extensive surface damage and collapse, suggesting that the site of PMP fungicidal action may directly or indirectly involve the fungal cell envelope.

Fungi are important intravascular pathogens. For example, *Candida* species cause widespread intravascular and visceral infections following hematogenous dissemination (6); also, *Candida* species are the most common cause of fungal endocarditis (15). Such intravascular fungal infections result from a complex interaction among platelets, phagocytes, plasma factors, endothelium, and the organism itself (3, 13). As has been shown for *Candida* species (13, 14), platelets may serve an important protagonistic function in the development of intravascular infection via fungus-platelet adherence at sites of endothelial damage. In contrast, recent studies have suggested that platelets may also serve an antagonistic function against intravascular infections in general via the release of microbicidal proteins from α-granules following thrombin stimulation (26). One such antimicrobial protein, which has significant in vitro bactericidal activity against known endocarditis pathogens, such as viridans group streptococci (4) and *Staphylococcus aureus* (26), has been termed platelet microbicidal protein (PMP [26]). PMP has been partially purified from thrombin-stimulated platelet extracts, has been found to have a low molecular mass (~8.5 kDa), and has been shown to be cationic and structurally as well as microbiologically distinct from lysozyme (26). Of note, *S. aureus* isolates from patients with confirmed endocarditis are more frequently resistant to PMP than are isolates from patients with *S. aureus* bacteremia in the absence of endocarditis (25). These findings suggest that PMP resistance may be one microbial virulence factor that is important in the induction and development of intravascular infections such as endocarditis. In the present study, we investigated the in vitro effects of PMP on the survival and ultrastructure of two clinically important yeast pathogens, *Candida* and *Cryptococcus* species.

MATERIALS AND METHODS

Organisms. The following five species of *Candida* were studied: *C. albicans* (six strains: ATCC 36082, B311, and four clinical isolates) as well as one clinical isolate each of fluconazole-susceptible and -resistant *C. glabrata* (obtained from E. M. Johnson, Bristol Royal Infirmary, Bristol, United Kingdom), *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*. Six distinct *Cryptococcus neoformans* isolates were studied, including ATCC 36556 (encapsulated), ATCC 52817 (nonencapsulated), and four clinical strains. All *Candida* and *Cryptococcus* clinical strains used in the present study were blood culture isolates from the Clinical Microbiology Laboratory of Harbor-UCLA Medical Center. All fungal isolates were routinely stored on Sabouraud dextrose agar slants at 4°C. For experimentation, organisms were grown for 16 to 18 h in yeast-nitrogen base (YNB) broth supplemented with amino acids (Difco Laboratories, Detroit, Mich.) and enriched with 0.5% (wt/vol) glucose at 27°C on a rotating drum. After harvesting by centrifugation (3,000 × g, 10 min), the yeast cells were resuspended in normal saline (0.85% NaCl), sonicated (60 Hz; sonifier model 350; Branson, Danbury, Conn.) for 4 s to ensure singlet organisms, washed twice in normal saline, and sonicated again. Washed, sonicated yeast cells were then counted in a hemacytometer and adjusted to the desired concentration (number of yeasts per milliliter) in Hanks' balanced salt

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solution containing calcium and magnesium (Irvine Scientific, Santa Ana, Calif.). Hemacytometer-determined yeast concentrations (CFU per milliliter) were confirmed by quantitative cultures on dilution plates.

Determination of amphotericin B MICs. The MIC of amphotericin B (E. R. Squibb and Sons, Inc., Princeton, N.J.) for representative *C. albicans* and *C. neoformans* strains was determined by a modification of the broth macrodilution method proposed by the Subcommittee on Antifungal Susceptibility Testing, National Committee for Clinical Laboratory Standards, in recent studies (18). For these determinations, RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing serial dilutions of amphotericin B (range, 0.117 to 60 μg/ml) was inoculated to achieve a final organism inoculum of 10^4 CFU/ml in 1-ml cultures within polystyrene plastic culture tubes (Becton Dickinson, Rutherford, N.J.). Cultures were then incubated for 48 h at 35°C in a nonhumidified, ambient CO_2 atmosphere. The MIC of amphotericin B for the *Candida* as well as the *C. neoformans* strains was defined as the lowest concentration which inhibited visible growth. The MICs that were obtained were subsequently used in studies comparing PMP and amphotericin B fungicidal activities.

Preparation of thrombin-induced PMP. PMP was prepared as described previously (26). Briefly, blood from New Zealand White rabbits was freshly collected into siliconized tubes containing citrate anticoagulant (9:1) and 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 PMP were subsequently produced from such washed platelet suspensions (10^8/ml) by stimulation with bovine-derived thrombin (1 U/ml, 37°C, 20 min; Sigma) in the presence of 0.2 M $CaCl_2$. Following thrombin-induced platelet activation, residual platelet material was removed by centrifugation and the PMP-rich supernatant was recovered. PMP-rich supernatants were pooled, dialyzed against 10 volumes of sterile deionized water in tubing with a molecular mass cutoff of 3.5 kDa (Spectrapor 3; Spectrum Medical Industries, Los Angeles, Calif.), and stored at −20°C until use.

Bioactivities of PMP preparations. The bioactivities of the thrombin-induced PMP preparations were determined by using techniques modified from those of Donaldson and Tew (5) and Weksler and Nachman (24). Briefly, bioassays were performed with *Bacillus subtilis* ATCC 6633, an indicator organism that is highly susceptible to the bactericidal action of PMP (27). For use in the bioassay, *B. subtilis* was grown in brain heart infusion broth (Difco Laboratories) at 37°C (14 h); organisms were harvested by centrifugation, washed twice in normal saline, and resuspended in phosphate-buffered saline (PBS; pH 7.2) prior to use. All PMP bioactivity assays were performed in low-protein-binding, 96-well microtiter plates (Corning Glass Works, Corning, N.Y.). *B. subtilis* at an inoculum of 10^4 CFU/ml was added to microtiter wells containing a range of dilutions of the PMP-rich preparation to achieve a final inoculum of 10^3 CFU/ml per well and a final range of PMP dilutions of 1:1 to 1:1,024 (final well volume, 200 μl); one well contained *B. subtilis* in MEM alone as a positive growth control. The microtiter plates were then incubated in ambient CO_2 (37°C). At 0, 15, and 30 min of incubation, 20-μl aliquots were removed from each well, diluted into PBS containing 0.01% (wt/vol) sodium polyanethol sulfonate (to inhibit further PMP-induced bacterial killing), sonicated, and quantitatively cultured onto 6.6% sheep blood agar. Killing curves were then constructed to determine percent *B. subtilis* survival over time. PMP bioactivity was defined as the inverse of the highest PMP dilution (in units per milliliter) which retained ≥95% lethality versus *B. subtilis* within the 30-min assay time period (26). The specific activity of PMP was then defined and quantified as units of PMP bioactivity per milligram of protein. Thrombin (1 U/ml) in PBS or MEM, as well as supernatants from washed platelets not exposed to thrombin, were used as additional controls in the PMP bioactivity assays.

PMP inactivation by anionic adsorption. Because of the cationic nature of PMP, microbicidal activity has been shown to be removable from PMP preparations by anionic adsorption (26). Inactive (control) PMP preparations were thus prepared by passage of bioactive thrombin-induced PMP preparations over a syringe-mounted anionic filter membrane (cellulose acetate/nitrate; Millipore Corp., Bedford, Mass.). The resulting filtrate exhibited no bactericidal activity when it was used in a bioassay against *B. subtilis* as described above.

Susceptibilities of *Candida* and *Cryptococcus* species to PMP. The susceptibilities of various *Candida* species and *C. neoformans* to PMP were assayed by exposing the organisms, at an inoculum of 10^4 CFU/ml in 0.2-ml volumes within siliconized glass tubes (Becton Dickinson), to PMP at a final concentration of 100 U/ml (specific activity, ~12.5 U/mg of protein). PMP-exposed cells were incubated at 27°C for times ranging from 0 to 180 min. At selected intervals, 20-μl samples were withdrawn, diluted into PBS containing 0.01% sodium polyanethol sulfonate, sonicated, and quantitatively cultured onto Sabouraud dextrose agar. Plates were then incubated at 37°C for 24 h (for *Candida* species) or 48 h (for *C. neoformans* isolates), and the surviving colonies were enumerated. Control cultures containing organisms suspended in MEM, Hanks' balanced salt solution, normal saline, or YNB alone or PMP preparations rendered inactive by anionic adsorption were performed in parallel and were processed identically. Results comparing organism survival in control versus PMP-exposed cultures were calculated and expressed as percent survival ± standard deviation. In all experiments, *B. subtilis* was assayed in parallel as a positive control for PMP bioactivity. For each isolate, a minimum of three PMP susceptibility assays were performed independently.

Confirmation of PMP fungicidal activity. Our previous studies (26) have revealed that the antistaphylococcal bactericidal activity within thrombin-induced platelet preparations is associated with a small (~8.5-kDa) cationic protein designated PMP. To confirm that this same protein was responsible for the observed fungicidal bioactivity of thrombin-induced PMP preparations, a series of compositional and functional experiments was performed. Thrombin-induced and anionic adsorption-inactivated PMP preparations were produced as described above. Following use in PMP inactivation steps, anionic membranes were eluted with an equivalent volume of 1.5 M NaCl, and the eluent was collected, dialyzed (molecular mass cutoff, 3.5 kDa; Spectrapor 3, Spectrum Medical Industries, Los Angeles, Calif.) against 100 volumes of 0.5% acetic acid, and concentrated by vacuum evaporation (SpeedVac; Savant Instruments, Farmingdale, N.Y.). Control 1.5 M NaCl elutions of anionic membranes not exposed to PMP preparations were performed in parallel. Samples of thrombin-induced and anionic

membrane-inactivated PMP preparations, as well as anionic membrane eluent and thrombin reagent alone (1 U/ml in MEM), were subjected to acid-urea or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10, 21) for composition and molecular mass analysis. Each sample was also tested for its microbicidal activity against *B. subtilis* ATCC 6633 as well as *C. albicans* 36082 as described above, with a minimum of four experimental replicates.

Relationship of PMP concentration to antifungal activity. The relative antifungal activities of various concentrations of PMP were assessed against *C. albicans* ATCC 36082 as well as *C. neoformans* ATCC 36556, each of which was cultured as described above. Yeast cells were added to siliconized glass tubes containing a range of PMP dilutions in Hanks' balanced salt solution to achieve a final inoculum of 10^4 CFU/ml and a final range of PMP concentrations of 10 to 150 U/ml. In parallel, control cultures contained either *C. albicans* or *C. neoformans* in corresponding concentrations of inactivated PMP preparations or in Hanks' balanced salt solution alone. Experimental cultures were incubated at 27°C for 120 min, at which time 20-μl samples were removed, diluted into PBS containing 0.01% sodium polyanethol sulfonate, and quantitatively cultured onto Sabouraud dextrose agar plates. Following incubation for 24 h (*C. albicans*) or 48 h (*C. neoformans*), colonies were enumerated and killing curves were constructed to compare percent yeast survival over time. Cultures of *B. subtilis* were assayed in parallel as a control for PMP bioactivity.

Relationship of inoculum size to PMP antifungal activity. In separate experiments, the effects of inoculum size on the antifungal activity of PMP were studied. PMP was added to yeast cells (washed, resuspended *C. albicans* ATCC 36082 or *C. neoformans* ATCC 36556) to achieve a final PMP concentration of 50 U/ml and a final organism inoculum of 5×10^3, 1×10^4, or 1×10^5 CFU/ml. At 0, 15, 30, 60, 120, and 180 min of incubation at 27°C, samples were withdrawn and subcultured as outlined above. Curves were then constructed to compare organism survival over time. In each experiment, PMP bioactivity was verified in parallel via assay of *B. subtilis*.

Comparison of PMP versus amphotericin B antifungal activity. For comparative purposes, survival curves of *C. albicans* ATCC 36082 and *C. neoformans* ATCC 36556 cultured in the presence of either PMP (50 U/ml) or 1× MICs of amphotericin B (0.47 μg/ml for both organisms) were determined in parallel, as described above. For each assay, fungal organisms were used at a final inoculum of 10^4 CFU/ml, and percent survival was monitored over a 180-min period.

Scanning electron microscopy. *C. albicans* ATCC 36082 and *C. neoformans* ATCC 36556 were grown in flasks containing 10 ml of YNB with and without sublethal concentrations of PMP (20 or 50 U/ml, respectively) on a rotating drum at 27°C for 18 h. Glutaraldehyde (Sigma) was then added to control and experimental cultures to yield a final fixative concentration of 1.5% (wt/vol). Following exposure to glutaraldehyde for 2 min, cells were harvested by centrifugation (3,000 × *g* at 4°C for 7 min) and were fixed overnight in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.25; Sigma) at 4°C. Specimens were then sequentially washed and resuspended in three changes of sodium cacodylate buffer for 1 h and were postfixed in 2% (wt/vol) osmium tetroxide (Polysciences, Inc., Warrington, Pa.) in sodium cacodylate buffer for 1 h. After washing for 30 min in two changes of sodium cacodylate buffer and one change of distilled water, samples were dehydrated in increasing concentrations of acetone (sequential range, 30 to 100%). One drop of yeast suspension prepared in this manner was then placed onto a clean, uncoated microscope coverslip (Corning Glass Works) and air dried. Each specimen was then mounted onto an aluminum specimen stub and coated with gold in a low-pressure argon atmosphere by using a sputter coater (Hummer IV Sputtering System; Technics Instruments, Alexandria, Va.) with a deposition angle of 45°C. Samples were then viewed by using a Hitachi S-405A (Hitachi, Tokyo) scanning electron microscope with a beam angle of 45°C and an accelerating voltage of 30 kV.

TABLE 1. Fungicidal and control bactericidal activities of experimental PMP preparations

Organism	% Survival[a]			
	Active PMP	Inactive PMP[b]	Eluent[c]	Thrombin[d]
C. albicans[e]	51.8 ± 5.8	84.7 ± 4.7	50.3 ± 3.2	99.3 ± 0.7
B. subtilis[f]	0.0 ± 0.0	84.1 ± 6.5	0.0 ± 0.0	88.3 ± 3.6

[a] Determined following 2 h of exposure using a 100 U/ml standardized thrombin-induced PMP-active preparation and other preparations as described in the text.
[b] Cellulose acetate or nitrate membrane inactivated (see text).
[c] Dialyzed eluent (1.5 M NaCl) from cellulose acetate/nitrate membrane used to prepare inactivated PMP (see text).
[d] Thrombin alone (1 U/ml of MEM).
[e] Tests with *C. albicans* were performed as described in the text by using an inoculum of 5×10^4 CFU/ml.
[f] Tests with *B. subtilis* were performed as described in the text by using the standard inoculum of 10^3 CFU/ml.

Statistical analyses. Quantitative results of multiple, independent experiments comparing the survivals of different organisms under various experimental conditions were analyzed by using the Student's *t* test with Fisher's modification for noncorrelated means and were corrected for multiple comparisons. For these analyses, *P* values of ≤0.05 were considered to be significant.

RESULTS

PMP fungicidal activity. Similar to our previous data with *S. aureus* (26), compositional and fungicidal assays indicated that the small (~8.5-kDa) cationic protein (PMP) is in fact the bioactive fungicidal moiety within partially purified thrombin-induced PMP preparations. The results shown in Table 1 demonstrate that both thrombin-induced PMP and anionic membrane elution preparations each possess fungicidal as well as bactericidal activities, while neither thrombin alone nor anionic membrane-inactivated preparations exhibits significant bioactivity. For example, there was no significant difference in survival of *C. albicans* within the thrombin-induced PMP (100 U/ml) and anionic membrane elution preparations. In addition, there was no significant difference in *C. albicans* survival within preparations of inactivated PMP (mean survival, 84.7% ± 4.7%) or thrombin alone (1 U/ml of MEM; mean survival, 99.3% ± 2.8%). Importantly, killing of both *C. albicans* and *B. subtilis* by thrombin-induced or anionic membrane-eluted PMP preparations was significantly greater than that observed with either inactivated PMP or thrombin control preparations ($P < 0.05$). Eluents from anionic membranes not exposed to active PMP preparations revealed no antimicrobial bioactivity (data not shown). Moreover, SDS-PAGE (data not shown) and acid-urea–PAGE analyses revealed the presence of the same small protein (PMP) within the active thrombin-induced as well as the anionic membrane-eluted PMP preparations and

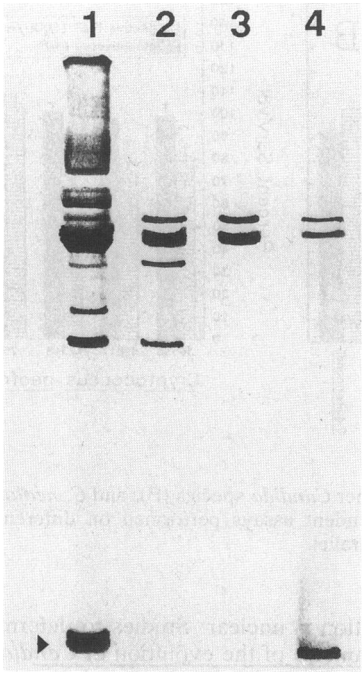

FIG. 1. Acid-urea–PAGE of fully bioactive thrombin-induced PMP (lane 1), anionic membrane-inactivated PMP (lane 2), thrombin reagent (lane 3), and bioactive eluent recovered from the anionic membrane used to inactivate PMP (lane 4). Only samples represented in lanes 1 and 4 were bioactive (fungicidal and bactericidal), while the samples shown in lanes 2 and 3 were neither fungicidal nor bactericidal (see text). The arrowhead indicates the presence of PMP in each of the bioactive samples (lanes 1 and 4) and its absence from nonbioactive samples (lanes 2 and 3).

its absence from anionic membrane-inactivated PMP and thrombin preparations (Fig. 1). The absence of this protein from samples consistently correlated with the loss of both bactericidal and fungicidal activities. Collectively, these data indicate that PMP is the bioactive moiety responsible for fungicidal activity within thrombin-induced platelet preparations.

Susceptibilities of *Candida* and *Cryptococcus* species to PMP. A summary of the susceptibilities of *Candida* and *C. neoformans* strains to PMP is shown in Table 2. In general, *Candida* species were more susceptible to PMP (mean survival, 33.6% ± 8.3%; Fig. 2A and 2B) than *C. neoformans* strains were (mean survival, 77.8% ± 6.9%; Fig. 2C).

TABLE 2. PMP susceptibilities of *Candida* species and *C. neoformans*[a]

Organism	No. of organisms[b]	Mean % survival
C. tropicalis	1	26.2 ± 4.8
C. albicans	6	38.7 ± 6.2
C. glabrata	2	51.2 ± 3.7
C. parapsilosis	1	99.2 ± 6.2
C. guillermondii	1	103.4 ± 7.1
C. neoformans	6	76.3 ± 8.2

[a] Determined by using an organism inoculum of 10^4 CFU/ml in 100 U of PMP per ml for 180 min.

[b] Each strain was assayed independently a minimum of three times.

The only exceptions to this trend were *C. guilliermondii* and *C. parapsilosis*, which were completely insusceptible to PMP (Fig. 2B). In this investigation, on the basis of extensive preliminary data, we defined a relative breakpoint for yeast PMP susceptibility as ≤50% survival of the initial inoculum (10^4 CFU/ml) after 120 min of exposure to the PMP preparation (100 U/ml). On the basis of this breakpoint, five of the six *C. albicans* isolates (with the exception of strain 17751) as well as the *C. tropicalis* isolate were found to be susceptible to PMP. In contrast, isolates of *C. glabrata* and *C. parapsilosis* as well as those of *C. guilliermondii* and all six *C. neoformans* isolates tested were relatively resistant to PMP-induced killing (survival range, 54.5% ± 4.7% to 100%). No *Candida* or *C. neoformans* isolates exposed to PMP preparations rendered inactive by anionic adsorption exhibited a significant decrease in CFU per milliliter within the 180-min assay period.

Relationship of PMP concentration to antifungal activity. The percent survival of a representative *C. albicans* strain (ATCC 36082; initial inoculum, 10^4 CFU/ml) following a 120-min exposure to various concentrations of PMP is shown in Fig. 3. In these studies, there was a clear correlation between increasing PMP concentration and decreasing *C. albicans* survival (linear correlation coefficient = 0.82; $P < 0.05$). Control cultures exposed in parallel to identical concentrations of inactivated PMP preparations exhibited no significant decrease in CFU per milliliter over the time period studied.

PMP antifungal activity at different inocula. Figure 4 depicts the antifungal activity of PMP against different inocula of *C. albicans* ATCC 36082 exposed to 50 U of PMP per ml. At inocula of 10^3 and 10^4 CFU/ml, PMP-induced *C. albicans* killing was time dependent, with the greatest observed reduction in CFU per milliliter (survival, 32.7% ± 5.2%) occurring at 180 min (Fig. 4A and B). Although PMP-induced *Candida* killing occurred at each of the inocula tested (5×10^3, 1×10^4, and 1×10^5), increasing inocula resulted in a corresponding decrease in killing (74.6% ± 4.4% survival with an initial inoculum of 10^5 CFU/ml; Fig. 4C). Similar PMP-induced antifungal activities were also observed for *C. neoformans* ATCC 36556 (data not shown). However, in comparison with the fungicidal effect of PMP on *C. albicans*, PMP exerted a substantially diminished fungicidal effect on *C. neoformans* (final inoculum, 10^5 CFU/ml), with little killing occurring within 180 min when 50 U of PMP per ml was used.

Comparison of PMP versus amphotericin B antifungal activity. A comparison of the activities of PMP (50 U/ml) and amphotericin B (1× the MIC; 0.47 μg/ml) demonstrated that PMP is nearly equivalent to amphotericin B in killing *C. albicans* ATCC 36082 (survival, 21.5% ± 6.3% and 19.1% ± 6.7%, respectively, after 180 min of exposure [no significant difference; $P \leq 0.32$]; Fig. 5). The killing of *C. albicans* by both amphotericin B and PMP was significantly greater than that achieved by inactivated PMP ($P \leq 0.05$ and $P \leq 0.01$, respectively; data not shown). At 1× the MIC, amphotericin B was superior to PMP in reducing the number of surviving *C. neoformans* ATCC 36556 CFU per milliliter within the 180-min test period, with survivals of 21.6% ± 5.9% and 70.1% ± 8.2%, respectively ($P \leq 0.05$; Fig. 4).

Effects of PMP on fungal surface structure. The effects of PMP on fungal surface structure are shown in Fig. 6 and 7. Control *C. albicans* ACTT 36082 cells unexposed to PMP exhibited a smooth-walled appearance and were spherical to ovoid in shape (Fig. 6A). In contrast, *C. albicans* ATCC 36082 cells grown in sublethal concentrations of PMP (20

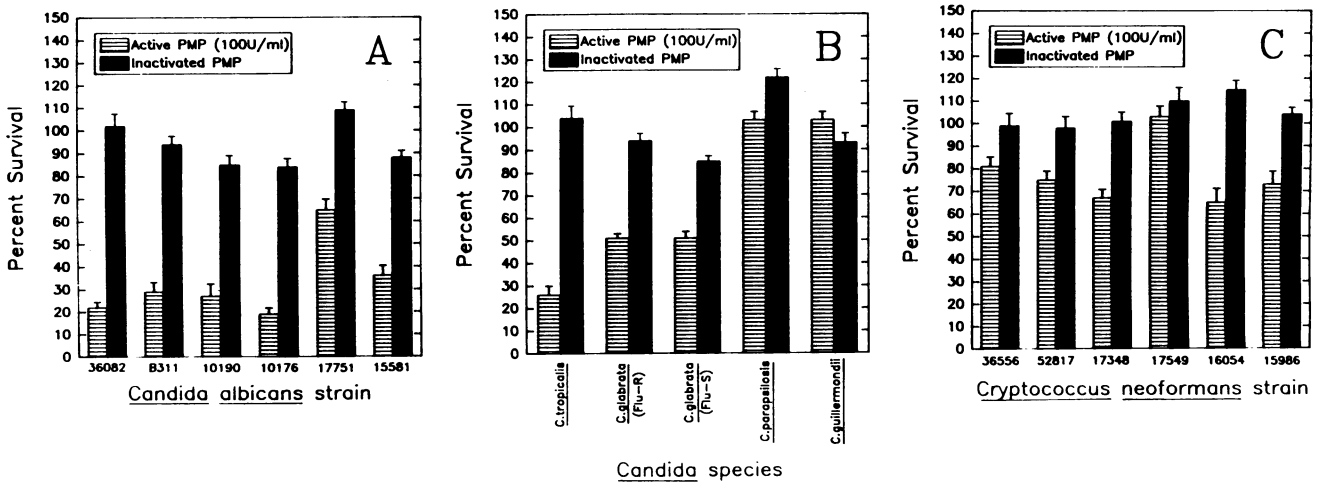

FIG. 2. Antifungal activity of PMP (100 U/ml, 180 min) against *C. albicans* (A), other *Candida* species (B), and *C. neoformans* (C). Each histogram represents the results ± standard error of a minimum of three independent assays performed on different days. Anionic membrane-inactivated PMP suspensions, which served as controls, were tested in parallel.

U/ml) appeared to be damaged, with extensive outer surface deformation (Fig. 6B). As observed in *Candida* cells, *C. neoformans* cells not exposed to PMP had smooth, spherical outer surfaces (Fig. 7A). However, unlike *Candida* cells, there were no detectable morphological alterations seen in cryptococcal organisms as a result of growth for 24 h in the presence of 20 U of PMP per ml. However, increasing the PMP concentration to 50 U/ml did lead to a demonstrable structural effect on cryptococcal cells (Fig. 7B), resulting in extensive cell damage and collapse, with some cells exhibiting concavity, which is consistent with the loss of cell envelope integrity (8).

DISCUSSION

The interaction between platelets and unicellular fungal pathogens in the initiation and development of intravascular

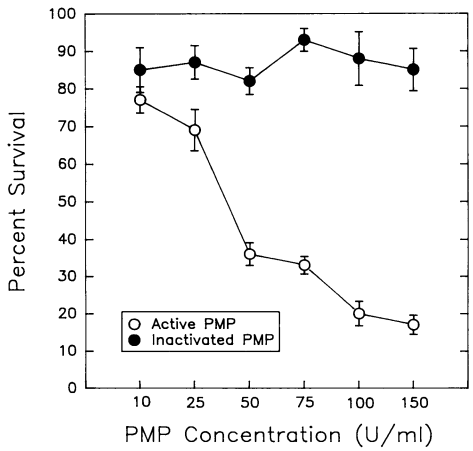

FIG. 3. Dose-response relationship of PMP versus *C. albicans* ATCC 36082. As described in the text, the organism inoculum (10^4 CFU/ml), PMP exposure time (180 min), and incubation temperature (27°C) were held constant in the experiments, while only the PMP concentration varied. Each point represents a minimum of three independent results ± standard error.

fungal infection is unclear. Studies to determine the pathogenic mechanisms of the evolution of *Candida* endocarditis have demonstrated that these organisms preferentially attach to platelet-fibrin matrices on the valve surface and that fungal viability is essential for such adherence (3, 13, 14). Moreover, these studies determined that *C. albicans* and *Candida stellatoidea* adhere to platelet-fibrin matrices more avidly than do other species of *Candida* or *Saccharomyces cerevisiae* (13). In addition, Robert et al. (20) have described the molecular interaction between platelets and *Candida* species to be mediated by the platelet glycoprotein IIb/IIIa (fibrinogen receptor) as well as by multifunctional adhesins on the candidal surface. To date, there is little information concerning the interaction of *C. neoformans* with platelets.

A number of bioactive proteins are secreted by mammalian platelets in response to activation, aggregation, or immune injury-induced degranulation (23). The majority of such secretory proteins serve to modulate hemostatis (e.g., fibrinogen) or serve as mediators of platelet adherence to the vascular endothelium (e.g., thrombospondin [22]). Antibacterial activity has also been associated with supernatants derived from activated platelets. A number of investigators have studied β-lysins, a class of heat-stable, antibacterial proteins recovered from platelets stimulated by various agonists (5, 23); the bactericidal spectra of such β-lysins include *B. subtilis* as well as several *S. aureus* isolates. Our laboratory has recently demonstrated (26) that thrombin stimulation of rabbit platelets induces the release of an α-granule-derived antimicrobial protein, which has been called platelet microbicidal protein, or PMP. PMP has been shown to be bactericidal against a significant percentage of both laboratory and clinical *S. aureus* isolates (25, 26) and may aid in preventing bacterial adherence to sterile thrombotic vegetations (27). The antifungal activities of PMP have not been investigated previously. In the present study, we examined the effect of thrombin-induced PMP on the in vitro survival and ultrastructure of two important intravascular fungal pathogens, *Candida* species and *C. neoformans*. Several interesting observations emanated from the investigation.

PMP possessed variable fungicidal activity against laboratory and clinical strains of several species of *Candida*; in

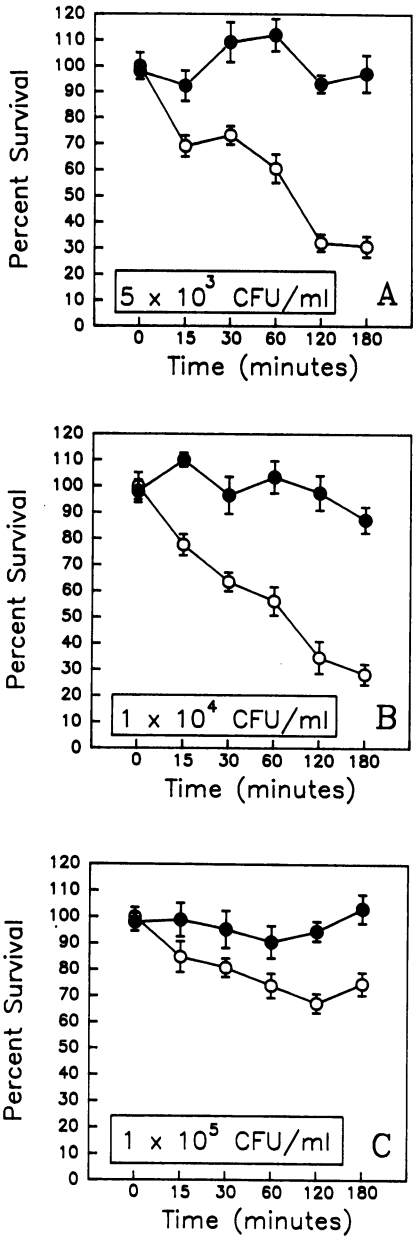

FIG. 4. Relationship between *C. albicans* 36082 inoculum and PMP antifungal activity. Active PMP (50 U/ml; ○) as well as control, inactivated PMP (●) were tested against various fungal inocula: 5×10^3 CFU/ml (A), 1×10^4 CFU/ml (B), and 1×10^5 CFU/ml (C). All experiments were performed at 27°C and were monitored over 180 min, as indicated. Each point represents a minimum of three independent results ± standard error.

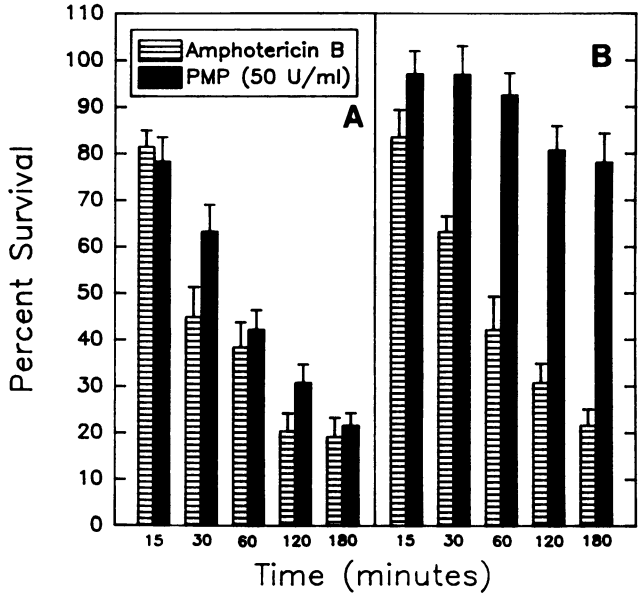

FIG. 5. Comparison of amphotericin B (1× the MIC; see text) versus PMP (50 U/ml) against *C. albicans* 36082 (A) and *C. neoformans* 36556 (B). All experiments were performed at 27°C, and percent survival for each organism was determined from quantitative subcultures taken at the indicated times. Each histogram represents data from a minimum of three independent experiments ± standard error performed on different days.

contrast, PMP had low fungicidal activity against clinically relevant isolates of *C. neoformans*. The relative potency of PMP against these pathogens exhibited a rank order of *C. tropicalis* and *C. albicans* and then *C. glabrata*; *C. guilliermondii* and *C. parapsilosis*, as well as *C. neoformans*, were relatively resistant to killing by PMP. The fungicidal action of PMP versus *C. albicans* was directly dependent on the PMP concentration and time of exposure and was inversely related to the fungal inoculum tested. Of interest, the greater in vitro antifungal activity of PMP against *C. albicans* compared with that against *C. neoformans* was found to be parallel on scanning electron microscopic examination of these same organisms following exposure to sublethal PMP concentrations. Exposure to similar concentrations of PMP (20 U/ml) caused extensive cell surface damage to *C. albicans*, while *C. neoformans* cell surface damage required a higher PMP concentration (50 U/ml). Of importance, the antifungal activity of PMP was shown by anionic resin adsorption and gel electrophoresis to reside within a small (~8.5-kDa) cationic peptide, which was also bactericidal against *B. subtilis*. These data collectively confirm that the same low-molecular-mass protein (PMP) that we have previously shown (26) to be the bactericidal moiety is also responsible for the antifungal activity of thrombin-induced PMP. Detailed structural analyses of this peptide are being completed.

The underlying reasons for the observed distinct differences in PMP susceptibility among various *Candida* species are not known; however, the fundamental structural features of both PMP (small size, cationic charge) and the *Candida* cell envelope may provide some insights in this regard. Significant differences in cell wall composition and structure exist among *Candida* species (17). In particular, the mannan side chains in different *Candida* species vary in carbohydrate residue, length, and bonding, which collectively result in antigenic variation (28). Although the mechanism of PMP fungicidal action is unknown, other similar cationic antimicrobial proteins (e.g., defensins [7]) are known to interact with microbial cell membranes, leading to membrane perturbation and eventual cell death (9, 11, 12). Considering the cationic nature of PMP (26), it is conceivable that organisms possessing a relatively greater anionic charge within their cell envelopes would be differentially susceptible to PMP-induced killing than would organisms that have a relatively

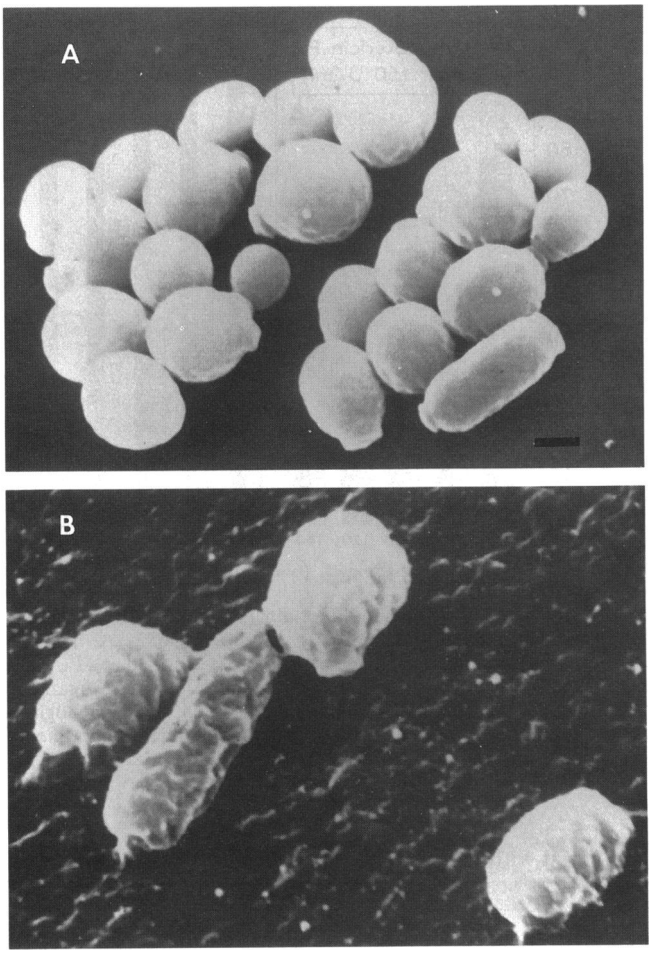

FIG. 6. Scanning electron micrographs illustrating the effect of PMP exposure on the surface integrity of *C. albicans* ATCC 36082. The yeasts (initial inoculum, 10^4 CFU/ml) were suspended in YNB for 18 h at 27°C with inactivated PMP (A) or active PMP (20 U/ml) (B). Note that organisms exposed to inactive PMP have a smooth surface (A), while those exposed to active PMP appear to have significant surface damage (B). Bar, 5 μm.

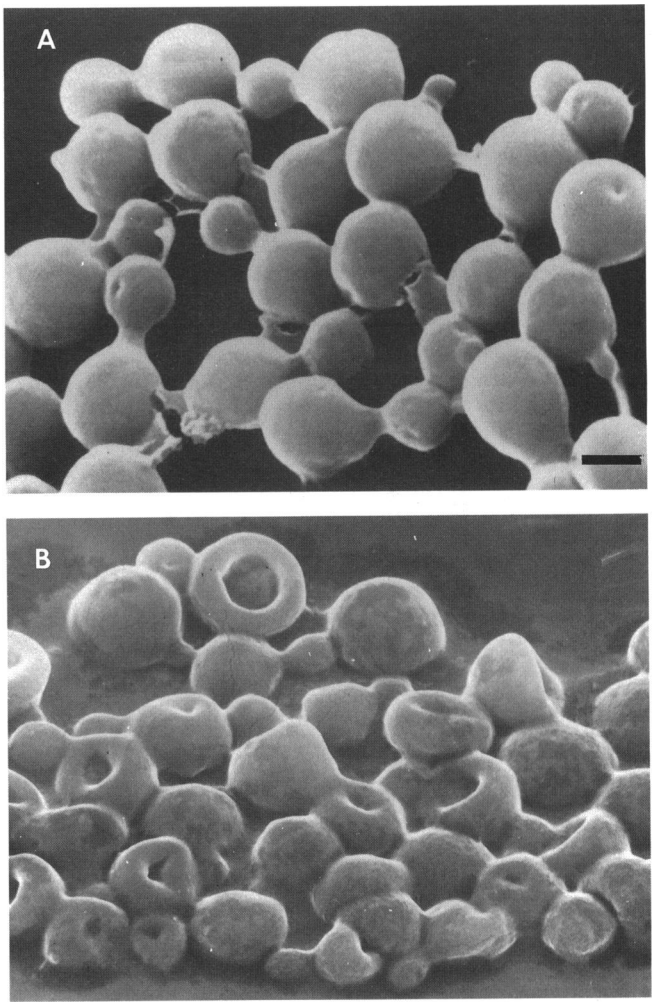

FIG. 7. Scanning electron micrographs illustrating the effect of PMP exposure on the surface integrity of *C. neoformans* ATCC 36556. The yeasts (initial inoculum, 10^4 CFU/ml) were suspended in YNB for 18 h at 27°C with inactivated PMP (A) or active PMP (50 U/ml) (B). Organisms exposed to inactivated PMP (A) appear smooth and spherical, while those exposed to active PMP have surface damage and collapse (B). Bar, 5 μm.

greater cationic surface charge, since the increased quantity of anionic charge may create a trapping "sink" for the cationic PMP. Furthermore, although *C. albicans* is known to possess two distinct permease systems for peptide uptake (19), there is no information regarding differences in PMP uptake via this pathway in relatively PMP-susceptible versus relatively PMP-resistant *Candida* species. Thus, *C. guillermondii* as well as *C. parapsilosis* may resist PMP-induced killing as a result of decreased PMP affinity or uptake or may use other potential mechanisms of resistance, such as fungal proteolytic degradation of PMP or alterations in membrane composition that result in reduced PMP interaction.

The mechanism(s) that accounts for the resistance to PMP observed in *C. neoformans* is also not understood. However, various serotypes of this organism are known to possess biochemically distinct polysaccharide capsules, typically composed of xylose, mannose, galactose, and glucuronic acid residues; glucuronoxylmannan is the most abundant polysaccharide constituent among *C. neoformans* serotypes A, B, C, and D (2). It is possible that such capsular material serves as a polyanionic sink, acting to inhibit the fungicidal action of cationic PMP. A similar phenomenon has previously been observed in mucoid *Pseudomonas aeruginosa* isolates which hyperproduce polyanionic alginate exopolysaccharide (1, 16); such organisms are less susceptible to the bactericidal action of cationic aminoglycoside antibiotics than their nonmucoid counterparts.

The increasing incidence of intravascular fungal infections provides a compelling motive for further investigations concerning the spectrum and mechanisms of fungicidal action of PMP and the mechanism(s) of fungal resistance to PMP. The relevance of our current observations concerning PMP in the host defense against endovascular fungal infections in vivo is not known; evaluation within discriminative experimental animal models will be critical in this regard. Moreover, no definitive information regarding similar microbicidal proteins from human platelets currently exists. Detailed studies addressing these points are under way in our laboratories.

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