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Evidence Implicating Phospholipase as a Virulence Factor of *Candida albicans*

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Three different approaches were used to investigate the role of extracellular phospholipases in the pathogenicity of *Candida albicans*. First, we compared 11 blood isolates of this yeast with an equal number of commensal strains isolated from the oral cavities of healthy volunteers. Blood isolates produced significantly more extracellular phospholipase activity than the commensal strains did. Second, two clinical isolates of *C. albicans* that differed in their levels of virulence in a newborn mouse model were compared for their ability to secrete phospholipases. The invasive strain produced significantly more extracellular phospholipase activity than the noninvasive strain did. Third, nine blood isolates were characterized for their phospholipase and proteinase production, germ tube formation, growth, and adherence to and damage of endothelial cells in vitro. These factors were analyzed subsequently to determine whether they predicted mortality in a mouse model of hematogenously disseminated candidiasis. By proportional hazard analysis, the relative risk of death was 5.6-fold higher (95% confidence interval, 1.672 to 18.84 [$P < 0.005$]) in the mice infected with the higher-phospholipase-secreting strains than in the low-phospholipase secretors. None of the other putative virulence factors predicted mortality. Characterization of phospholipases secreted by three of the blood isolates showed that these strains secreted both phospholipase B and lysophospholipase-transacylase activities. These results implicate extracellular phospholipase as a virulence factor in the pathogenesis of hematogenous infections caused by *C. albicans*.

Multiple characteristics of *Candida albicans* have been proposed to be virulence factors that enable the organism to cause hematogenously disseminated infections in susceptible hosts. These putative virulence factors include germination, adherence to host cells, and secretion of proteinases and phospholipases (reviewed in reference 6). Although there has been extensive research on putative virulence factors of *C. albicans*, no factor has been linked unequivocally with pathogenicity (6).

Although phospholipases contribute to the pathogenicity of bacteria (30), rickettsiae (24), and protozoa (21), the role of these membrane-damaging enzymes (22) in the pathogenicity of *C. albicans* is defined incompletely. Extracellular phospholipases that have been reported to be secreted by *C. albicans* include phospholipase A (2), phospholipase B (1), and phospholipase C (19). Also, the organism secretes lysophospholipase and lysophospholipase-transacylase (1, 25). Some of these different phospholipase activities may be contained within the same enzyme (15).

In the current study, we examined extracellular phospholipase activity of *C. albicans* as a potential virulence factor by (i) comparing the activities of these enzymes of commensal and blood isolates; (ii) quantifying the in vitro expression of putative virulence factors (including phospholipase) by two clinical isolates of *C. albicans* that differed markedly in their ability to cause disseminated infection in the newborn mouse model; and (iii) prospectively measuring the expression of putative virulence factors of blood isolates of *C. albicans* in vitro and then determining which of these factors were predictive of mortality in the murine model of hematogenously disseminated candidal infection. The data obtained from these experiments strongly suggested that phospholipases are important factors in the pathogenesis of *C. albicans*. Therefore, we further determined the amount and type of phospholipases secreted by three strains of *C. albicans* which differed significantly in their phospholipase secretion. Biochemical analysis showed that the main phospholipases secreted by *C. albicans* are phospholipase B and lysophospholipase-transacylase.

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MATERIALS AND METHODS

Reagents. Yeast nitrogen base broth with amino acids (YNB broth) and Sabouraud dextrose broth (SDB) were purchased from Difco Laboratories (Detroit, Mich.), and Hanks balanced salt solution (HBSS) with calcium and magnesium and RPMI 1640 medium were obtained from Irvine Scientific (Santa Ana, Calif.). Fetal bovine serum and M-199 medium were obtained from Gibco (Grand Island, N.Y.), and defined bovine calf serum was acquired from HyClone (Logan, Utah). The collagen matrix (Vitrogen) was purchased from Celtrix (Palo Alto, Calif.). Lysopalmitoyl phosphatidylcholine, labelled with L-1-(palmitoyl-1-^{14}C) (56.7 mCi/mmol), and phosphatidylcholine, labelled with L-α-dipalmitoyl-(dipalmitoyl-1-^{14}C) (115 mCi/mmol), were obtained from DuPont NEN (Boston, Mass.); 1-palmitoyl lysophosphatidylcholine and dipalmitoyl phosphatidylcholine

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were obtained from Funakoshi Chemical Company (Tokyo, Japan); and $Na_2{}^{51}CrO_4$ was obtained from ICN Biochemical (Irvine, Calif.).

Organisms. *C. albicans* ATCC 36082, a blood isolate, was obtained from the American Type Culture Collection (Rockville, Md.). *C. albicans* CA30 was isolated from a patient with vaginal candidiasis, while *C. albicans* CA87 was isolated from a patient with oral candidiasis. *C. albicans* 15153, 15563, 16240, 16427, 16653, 17236, 17737, and 18084 were blood isolates obtained from the Clinical Microbiology Laboratory at Harbor-UCLA Medical Center. *C. albicans* 1 to 11 were commensal isolates recovered from the oral cavities of healthy volunteers. The commensal isolates were identified with the API 20C identification system (Biomérieux, Marcy l'Etoile, France) and the criteria of germ tube formation in bovine serum. Working cultures were freshly prepared from stock cultures that were stored at −70°C (in 50% glycerol). Working cultures were kept at 4°C on Sabouraud dextrose agar (SDA) slants. Growth and harvesting of the organisms were performed as described previously (12). In all experiments, singlet blastospores were counted with a hemacytometer and adjusted to the desired concentration in either HBSS or RPMI 1640 medium, depending on the assay. The inoculum size was confirmed by quantitative culturing in SDA.

Growth studies. Ten-milliliter cultures of *C. albicans* grown overnight in YNB broth supplemented with 0.5% glucose were harvested, washed twice, resuspended in 0.85% NaCl, and added to flasks containing 50 ml of SDB to achieve a final inoculum of 10^5 yeast cells per ml. The flasks were incubated in a rotary shaker at 37°C. One-milliliter aliquots were removed at selected time points, and the optical density at 420 nm was measured. For comparison of the growth rates of different isolates, we used the optical density at 10 h because at this time point, the isolates showed the highest variation in their growth. After 24 h, all isolates grew to the same extent (data not shown).

Germ tube formation. The germination of various *C. albicans* strains was determined by a modification of our previously described method (11). Three milliliters containing 1.5×10^8 cells was added to an equal volume of bovine serum, and the samples were incubated on a rotary shaker at 37°C. At selected intervals, 0.5-ml samples were withdrawn and fixed in 0.5 ml of 2% (vol/vol) glutaraldehyde in distilled water. A wet mount from each strain was prepared, and the number of yeast cells with germ tubes, at least 1 blastospore diameter in length, was determined microscopically. At the same time, the germ tube lengths were measured with a micrometer. In these experiments, 100 organisms per field were examined. Each strain was tested in triplicate.

Determination of extracellular phospholipase activity by the egg yolk agar method. *C. albicans* strains were screened for production of extracellular phospholipase activity by growing them on egg yolk agar and measuring the size of the zone of precipitation by the method of Samaranayake et al. (23). Briefly, the egg yolk medium consisted of (in 184 ml of distilled water) SDA, 13.0 g; NaCl, 11.7 g; $CaCl_2$, 0.111 g; and 10% sterile egg yolk. The egg yolk was centrifuged at 500 × *g* for 10 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. A 10-μl suspension of 10^7 yeast cells per ml in saline was plated on the surface of the egg yolk medium in a 90-mm-diameter petri dish and left to dry at room temperature. The culture was then incubated at 37°C for 48 h, after which the diameter of the precipitation zone around the colony was determined. Each isolate was tested in replicates of three, and the experiment was carried out on two different occasions.

Characterization of phospholipase activity in culture supernatants. The types and amounts of phospholipases produced by three representative blood isolates of *C. albicans* were determined as described previously (1). The strains used were 18084, ATCC 36082, and 16240. These strains had low, intermediate, and high extracellular phospholipase activities, respectively, when tested by the egg yolk agar method. Each strain was grown in SDB at 37°C for 15 h on a rotary shaker. The cells were removed by centrifugation at 3,000 × *g* for 5 min, and the proteins in the culture filtrate (2 liters) were precipitated with ammonium sulfate. The solution was then stirred for 2 h and centrifuged at 16,000 × *g* for 15 min, and the resulting protein precipitate was collected. This protein was redissolved in buffer A (10 mM Tris HCl [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol) and dialyzed for 16 h against the same buffer. The standard incubation mixture for determination of phospholipase B activity consisted of [^{14}C]dipalmitoyl phosphatidylcholine (30,000 dpm) and carrier dipalmitoyl phosphatidylcholine (192.5 nmol) in 0.1% Triton X-100 and 0.1 ml of enzyme fraction, made up to a final volume of 0.25 ml with 0.1 M sodium citrate buffer (pH 4.0). This assay measured the rate of liberation of labelled fatty acid from [^{14}C]dipalmitoyl phosphatidylcholine (1). Incubation was carried out at 37°C for 60 min. The assay for phospholipase-transacylase measured the rate of production of labelled phosphatidylcholine from [^{14}C]palmitoyl lysophosphatidylcholine (25). [^{14}C]palmitoyl lysophosphatidylcholine (25,000 dpm) and 50 nmol of unlabelled palmitoyl lysophosphatidylcholine were suspended in 0.1 M sodium citrate buffer (pH 6.0), and enzyme solution was added to a final volume of 0.2 ml. The reaction was carried out at 37°C for 15 min.

For both assays, the reactions were stopped by adding 1.0 ml of a chloroform-methanol solution (1:2, vol/vol), and the reaction products were extracted by the method of Bligh and Dyer (3). To analyze the reaction products, the extract was evaporated and the residue was redissolved in an appropriate volume of chloroform-methanol (6:1, vol/vol). A sample of the solution was applied to a silica gel thin-layer plate. The plate was developed with chloroform-methanol-water (65:25:4, vol/vol/vol). Lipids were identified by comparing their R_f values with authentic standards (dipalmitoyl phosphatidylcholine, palmitoyl lysophosphatidylcholine, and palmitic acid). The silica gel areas corresponding to individual lipids were scraped off, and the radioactivity was determined in a liquid scintillation counter in 6 ml of toluene scintillator.

Phospholipase B activity and the hydrolysis activity of phospholipase-transacylase were expressed as nanomoles of free fatty acid released per milliliter per minute. The transacylation activity of the phospholipase-transacylase was expressed as nanomoles of phosphatidylcholine formed per milliliter per minute.

Determination of candidal proteinase activity. To measure the secretion of proteinase activity, *C. albicans* strains were grown in a synthetic vitamin solution by the method of MacDonald and Odds (16). The composition per 1 liter of distilled water was as follows: glucose, 20 g; bovine serum albumin, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; biotin, 20 μg; nicotinic acid, 400 μg; pyridoxal hydrochloride, 400 μg; riboflavin, 200 μg; and thiamine hydrochloride, 400 μg. Each organism was grown for 7 days in 250 ml of medium at 37°C on a rotary shaker. The yeast cells were then centrifuged at 5,000 × *g* for 30 min, after which the supernatant was passed through 0.45-μm-pore-size membrane filters to ensure the removal of any remaining organisms. The pH of the filtrate was adjusted to 6.5 with 3 M NaOH, and the proteinases were concentrated approximately 25-fold by ultrafiltration (Minitan-s, 10,000 normal molecular weight cutoff point [NMWL]; Millipore Co., Bedford, Mass.). Brij 35 solution (30%, wt/vol; Sigma) was added to the enzyme solution to a final concentration of 2% (vol/vol) to stabilize the enzyme, which was then sterilized by membrane filtration and stored in aliquots at −70°C.

The extracellular proteinase activity was determined by the method of MacDonald and Odds (16). Briefly, 0.5 ml of the enzyme solution was incubated with 2 ml of 1% (wt/vol) bovine serum albumin in 0.05 M sodium citrate buffer (pH 3.2) for 30 min. The reaction was stopped, and the undigested albumin was precipitated by adding 5 ml of 5% (wt/vol) trichloroacetic acid solution. The undigested albumin was removed by filtration (Whatman no. 3 paper), and the amount was quantified by the Bio-Rad Laboratories (Hercules, Calif.) protein assay. The extracellular proteinase activity was calculated by subtracting the amount of undigested albumin from the starting amount.

Pathogenicity of the blood isolates. The pathogenicity of blood isolates of *C. albicans* was tested by challenging 10 mice per isolate. Nine- to 11-week-old male, 18- to 20-g BALB/c mice (Harlan, San Diego, Calif.) were used in this study. Each mouse was injected with 0.5 ml of cell suspension (10^6 cells) in nonpyrogenic phosphate-buffered saline in the tail vein. The survival of the mice was monitored twice daily for 21 days. We chose 10^6 cells as a challenge inoculum because preliminary studies showed that 10^7 cells resulted in the death of all the animals within 48 h. In contrast, no death was observed when 10^5 cells were used as the inoculum at 7 days postchallenge.

The infant mouse model was used to study the virulence of strains CA30 and CA87 as described previously (4). Oral-intragastric inoculation (2×10^8 cells) was conducted with a 24-gauge feeding needle (Popper & Sons, Inc., New Hyde Park, N.Y.) attached to a 1.0-ml syringe. Cyclophosphamide (Adria Laboratories, Columbus, Ohio) and cortisone acetate (Merck Sharp & Dohme, West Point, Pa.) were used as immunocompromising drugs (4). Animals were sacrificed 20 days postchallenge by asphyxiation with CO_2 and prepared for histological examination. The tissue fixation, dehydration, and embedding procedures were the same as those described previously (4).

Preparation of human endothelial cells. Human umbilical vein endothelial cells were obtained by a modification of the method of Jaffe et al. (13). The cells were harvested with collagenase (Sigma) and grown in M-199 medium enriched with 10% fetal bovine serum, 10% defined bovine calf serum, L-glutamine, penicillin, and streptomycin. Second- or third-passage cells were grown to confluency in either 6-well or 24-well tissue culture plates (Costar, Van Nuys, Calif.) coated with a collagen matrix. All incubations were in 5% CO_2 at 37°C.

Adherence of *C. albicans* to endothelial cells. All adherence experiments were carried out in six-well tissue culture plates by a modification of our previously described method (11). Only confluent endothelial cells were used to avoid possible adherence of *C. albicans* cells to plastic surfaces. After rinsing the endothelial cell monolayers twice with prewarmed HBSS, 1.0 ml of the singlet blastospore suspensions (3×10^2 cells) in HBSS was added to each well. The inoculum size of each suspension was confirmed by quantitative culturing in SDA. The plate was incubated at 37°C for 30 min, after which the nonadherent organisms were aspirated and the endothelial cell monolayers were rinsed twice with 10 ml of HBSS in a standardized manner. A 1.5-ml volume of SDA was added to each well and allowed to solidify. After incubating the plate at 37°C for 24 h, the number of adherent organisms was determined by colony counting. Each isolate was tested in replicates of three, and adherence was expressed as the percentage of the inoculum added (mean ± standard deviation).

Endothelial cell damage. The ability of *C. albicans* strains to damage endothelial cells was determined by a modification of the ^{51}Cr release assay described previously (12). Endothelial cells were grown in 24-well tissue culture plates. The cells were incubated with $Na_2{}^{51}CrO_4$ in M-199 medium (2.5 μCi per well) for 16 h, after which the unincorporated tracer was aspirated and the wells were washed three times with prewarmed HBSS. The endothelial cells were infected with 10^5 organisms per well in 1 ml of RPMI 1640 medium and incubated at 37°C for 3 h. The spontaneous release of ^{51}Cr was determined by incubating endothelial cells in RPMI 1640 medium without organisms. Following incubation, 0.5 ml of medium was aspirated from each well and transferred to glass tubes for determination of ^{51}Cr activity. The endothelial cells were lysed with 6 N NaOH for 30

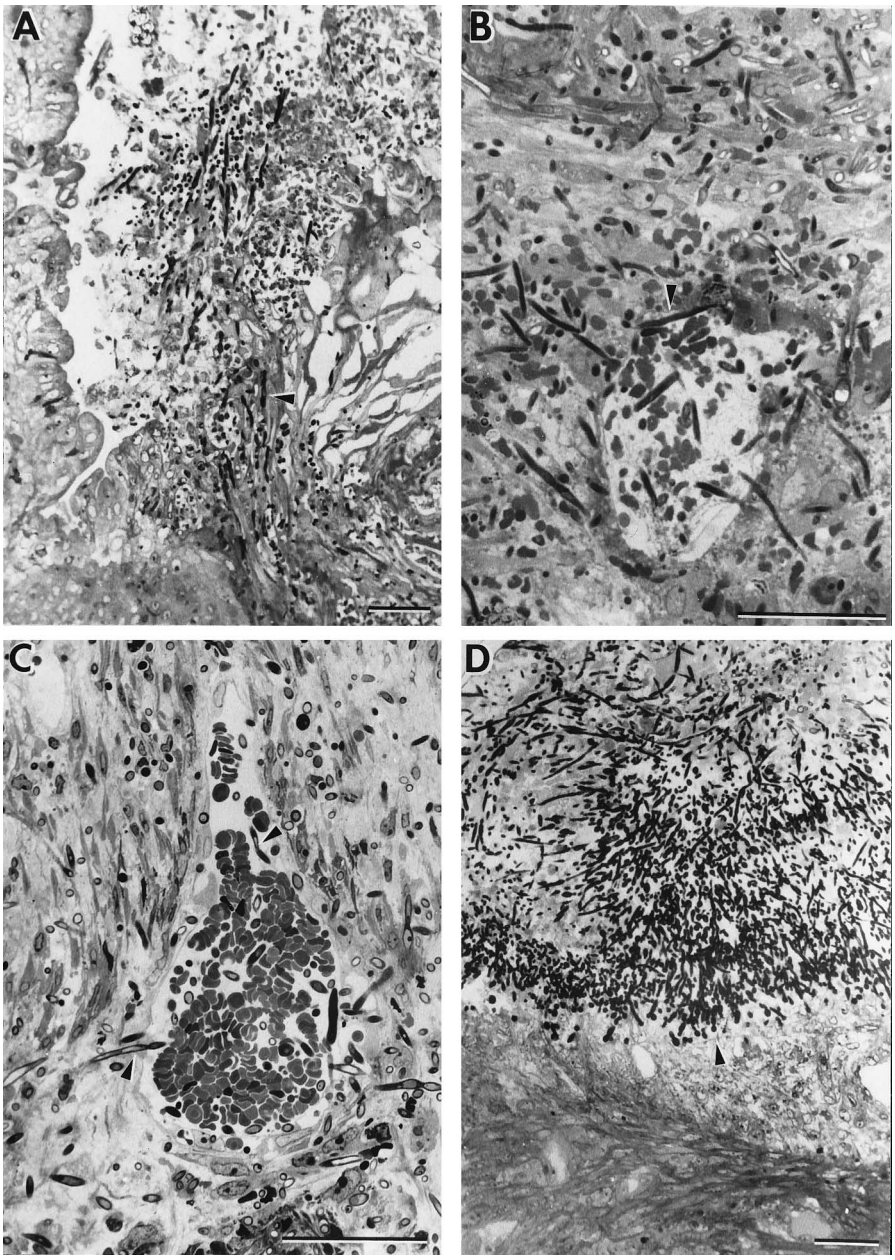

FIG. 1. Comparison of the infectivities of strains CA30 and CA87. (A) CA30-infected mouse stomach at junction of glandular and keratinized epithelium showing invasive elements (hyphal and yeast forms). Magnification, ×240. (B) CA30-invaded stomach showing multiple elements within the host tissue. Magnification, ×570. (C) Same as panel A, but showing elements in blood vessels. Magnification, ×570. (D) CA87-infected mouse stomach showing noninvasive fungal elements at mucosal surface. Magnification, ×240. Arrowheads indicate fungal elements.

TABLE 1. Comparison of blood and commensal isolates of *C. albicans* by phospholipase production and germination[a]

Type of isolates (*n*)	Phospholipase produced [(1/Pz) − 1][b]	Germ tube length (μm)	% Germination after 2 h
Blood (11)	0.30 ± 0.24	16.1 ± 4.0	93.0 ± 1.6
Commensal (11)	0.07 ± 0.05	9.6 ± 5.7	73.0 ± 22.7
P	0.008	0.015	0.031

[a] All values (except *P*) are means ± standard deviations.
[b] The phospholipase zone (Pz) is calculated by dividing the colony diameter by the cloudy-zone-plus-colony diameter.

min and removed from the wells by rinsing twice with 10% RadiacWash (Atomic Products, Shirley, N.Y.). The NaOH and the RadiacWash treatments were combined, and the amount of ^{51}Cr was measured with a gamma counter. The total amount of ^{51}Cr incorporated by the endothelial cells in each well equaled the sum of the radioactive counts per minute of the aspirated medium plus the radioactive counts of the endothelial cells lysed with NaOH and RadiacWash. After correcting the data for variations in the amount of tracer incorporated into each well, the percentage of specific endothelial cell release of ^{51}Cr was calculated by the following formula: [(experimental release × 2) − (spontaneous release × 2)]/[(total incorporation) − (spontaneous release × 2)]. Each experimental condition was tested in replicates of three.

Statistical analysis. The Cox proportional hazard method was used to determine the risk of death associated with each of the putative virulence factors. Correlation between each of the putative virulence factors was determined by Spearman's rank sum test. A P of <0.05 was considered significant.

TABLE 2. Comparison of putative virulence factors of *C. albicans* CA30 (invasive) and CA87 (noninvasive)

Strain	Tissue invasion	% Phospholipase production[a]	Proteinase production[b]	Growth rate (OD at 10 h)[c]	Endothelial cell adherence (%)[b]	% ^{51}Cr release[b]	% Germination[b]	Germ tube length (μm) after 2 h[b]
CA30	+	100	4.5 ± 0.11	0.96	40.0 ± 7.5	49.2 ± 4.7	94.0 ± 2.6	6.3 ± 1.5
CA87	−	27.9	4.7 ± 0.53	1.05	38.1 ± 8.0	59.9 ± 3.5	94.3 ± 2.6	6.8 ± 1.6

[a] Relative to that of CA30.
[b] Values are means ± standard deviations.
[c] OD, optical density.

RESULTS

Blood isolates of *C. albicans* produced higher extracellular phospholipase activities and germinated more readily than commensal isolates. We compared the extracellular phospholipase activities and germination of 11 blood isolates with those of 11 commensal isolates of *C. albicans*. Marked differences among strains from both sources were observed (Table 1). Compared with the commensal isolates, the blood isolates produced greater extracellular phospholipase activity ($P = 0.008$), had a higher rate of germination ($P = 0.03$), and produced longer germ tubes ($P = 0.016$).

An isolate with high extracellular phospholipase activity was invasive in the infant mouse model, whereas the isolate with low extracellular phospholipase activity was not. We next examined the virulence of two clinical isolates of *C. albicans*, strains CA30 and CA87, by use of the infant mouse model. We found that CA30 invaded through the gastric mucosa (Fig. 1) and disseminated hematogenously to other organs. In contrast, although the stomachs of the animals inoculated with CA87 were colonized, there was no invasion of the gastric mucosa (Fig. 1D) and no hematogenous dissemination. We have examined 28 clinical strains of *C. albicans* for their ability to cross the bowel wall of mice challenged by the oral-intragastric route as described below. Five of the 28 strains showed the same inability to cross the bowel wall as CA87 (unpublished data). Thus, CA87 is a representative strain of this naturally occurring, noninvasive group of clinical isolates. Likewise, CA30 is a representative strain of the more typical invasive group of clinical isolates.

To determine why CA30 was more virulent than CA87, the extracellular phospholipase and proteinase activities, levels of endothelial cell adherence and damage, rates of germination, and average germ tube lengths of the two strains were compared in vitro (Table 2). The more virulent strain, CA30, produced threefold more extracellular phospholipase activity than did CA87.

The blood isolates with high extracellular phospholipase activity caused increased mortality in the mouse model. We prospectively examined nine of the blood isolates for expression of virulence factors (Table 3). The mortality of mice infected with each of these isolates was determined, and the predictive value of each virulence factor for mortality was determined by Cox proportional hazard analysis (Table 4). Virtually all of the blood isolates germinated after 2 h of incubation in serum (data not shown); therefore, the percent germination of these isolates was not included in the analysis. Of the virulence factors studied, only extracellular phospholipase activity was predictive of mortality (Fig. 2). Moreover, there was no significant correlation among any of the virulence factors tested (data are not shown).

The extracellular phospholipase activity of three blood isolates of *C. albicans* consisted of phospholipase B and lysophospholipase-transacylase. Since we determined that the total extracellular phospholipase activity of the blood isolates predicted mortality, we evaluated which types of phospholipases contributed to the overall activity. We assayed culture filtrates for the activities of specific phospholipases. We tested three representative blood isolates, i.e., strain 18084, which had low extracellular phospholipase activity, strain ATCC 36082, which had intermediate activity, and strain 16240, which had high activity. All three strains secreted phospholipase B (Table 5) and lysophospholipase-transacylase. The phospholipase B activity of the isolates correlated well with their total extracellular phospholipase activity as determined by the egg yolk agar method. However, the hydrolysis activity of the lysophospho-

TABLE 3. Putative virulence factors of the blood isolates tested in vitro and in vivo

Strain	Phospholipase produced [(1/Pz) − 1][a]	Amt of proteinase produced (U)[b]	% Adherence[c]	% ^{51}Cr release[d]	Growth rate (OD at 10 h)[e]	Germ tube length (μm)	Median no. of surviving mice
ATCC 36082	0.35	54.3	37	100	0.788	15	8
15153	0.11	142.9	29.2	109.9	0.555	14.4	13
15563	0.06	178.9	37.5	28.8	0.649	22.5	7
16240	0.75	284.5	50.2	132.9	0.745	15.5	6
17236	0.59	261.4	28.4	62.8	0.848	17.5	7
17737	0.59	160.7	28.1	97.5	0.484	11.8	2
16427	0.33	280	35.9	101.6	0.690	19.7	4
16653	0.89	145	15.4	74.3	0.633	15.1	4
18084	0.09	91.5	32.2	60.6	0.734	17.8	2
Mean	0.42	177.7	32.7	85.4	0.681	16.6	
SD	0.3	82.2	9.4	31.5	0.114	3.2	

[a] The phospholipase zone (Pz) is calculated by dividing the colony diameter by the cloudy-zone-plus-colony diameter.
[b] Units are defined as the proteinase activity required to degrade 1 μg of bovine serum albumin in 45 min.
[c] Percent adherence is the number of yeast cells adhering to the endothelial cell monolayer.
[d] Percent ^{51}Cr release represents endothelial cell injury, calculated as a percentage of that of strain ATCC 36082.
[e] OD, optical density.

TABLE 4. Correlation between putative virulence factors of *C. albicans* and pathogenicity in mice

Covariate	Coefficient	Hazard	*P*	95% Confidence limits
Phospholipase	1.725	5.612	0.005	1.672, 18.84
Proteinase	−0.003	0.997	0.192	0.993, 1.00
Adherence[a]	1.748	5.743	0.448	0.063, 523.9
% ^{51}Cr release[b]	−0.616	0.540	0.572	0.064, 4.57
Growth rate (OD[c] at 10 h)	−1.562	0.209	0.185	0.021, 2.11
Germ tube length (μm)	0.048	1.050	0.720	0.808, 1.36

[a] Adherence of *C. albicans* to the endothelial cell monolayer.
[b] Percent ^{51}Cr release represents damage to endothelial cells.
[c] OD, optical density.

lipase-transacylase did not. Therefore, the egg yolk agar method detects mainly phospholipase B activity.

DISCUSSION

Extracellular phospholipases have been implicated as pathogenicity factors for several organisms such as *Clostridium perfringens* (26), *Rickettsia* spp. (24), *Toxoplasma gondii* (21), *Entamoeba histolytica* (20), *Listeria monocytogenes* (10), and *Staphylococcus aureus* (17). The type of phospholipases implicated in virulence varies with the organism. For example, *C. perfringens* (26) secretes a phospholipase C, whereas *T. gondii* (21) secretes a phospholipase A_2. Extracellular phospholipases facilitate the ability of these organisms to injure, invade, and/or egress from various host cells (21, 24, 30). Also, phospholipases C and A_2 may remove processed antigens from the surface of antigen-presenting cells (7, 8). The secretion of phospholipases by *C. albicans* was first detected by Costa et al. (5) and confirmed by Werner (28). *C. albicans* is the only *Candida* species known to secrete phospholipases (9, 23).

In the current study, blood isolates secreted more phospholipases than commensal strains did. This finding suggests that production of extracellular phospholipases may be a virulence factor for blood-borne infections. These blood isolates also expressed other virulence factors (e.g., germ tube formation and germ tube length) to a greater extent than the commensal isolates did. The ability of clinical isolates to constitutively express a variety of virulence factors at a higher level than that

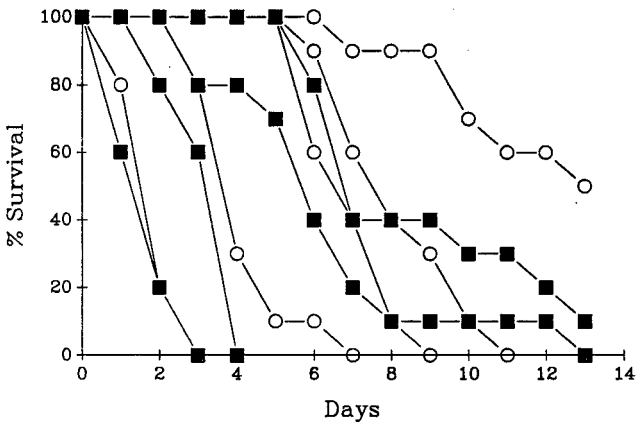

FIG. 2. Survival of mice challenged intravenously with 10^6 blastospores of blood isolates of *C. albicans* that secreted low (○) or high (■) levels of phospholipase.

TABLE 5. Phospholipase B and lysophospholipase-transacylase activities in three strains of *C. albicans* that varied in phospholipase secretion[a]

Strain	Phospholipase produced [(1/Pz) − 1][b,c]	Phospholipase B activity (nmol/ml/min)[c]	Lysophospholipase-transacylase activity (nmol/ml/min)[c]	
			Transacylation	Hydrolysis
18084 (weak)[d]	0.09 ± 0.4	1.1 ± 0.4	55 ± 13	5.0 ± 1.1
ATCC 36082 (moderate)	0.34 ± 0.1	3.2 ± 1.0	147 ± 35	3.2 ± 1.0
16240 (potent)	0.75 ± 0.12	4.5 ± 1.3	293 ± 58	4.5 ± 1.3

[a] The assays were performed on cultures grown for 9 h.
[b] The phospholipase zone (Pz) is calculated by dividing the colony diameter by the cloudy-zone-plus-colony diameter.
[c] Values are means ± standard deviations.
[d] Weak, moderate, and potent indicate the ability of the strain to secrete phospholipase.

of commensal isolates has been reported for *Pseudomonas aeruginosa* (14).

Further evidence that production of extracellular phospholipase was associated with the development of invasive disease was provided by the finding that the only apparent difference between *C. albicans* CA30 (invasive strain) and CA87 (noninvasive strain) was in the enhanced production of extracellular phospholipase by CA30. Although it was not invasive, the low-phospholipase-secreting strain, CA87, was still able to colonize the gastric mucosa. These results confirm our finding with the commensal isolates that secretion of phospholipases is not requisite for mucosal colonization.

To prospectively determine which virulence factors were important in the development of hematogenous infections in vivo, we examined the in vitro expression of selected virulence factors of blood isolates of *C. albicans* and correlated these factors with survival in the mouse model. We found that only the production of extracellular phospholipase was predictive of mortality. Similarly, Barrett-Bee et al. (2) examined the relationship between extracellular phospholipase production, epithelial cell adherence, and mortality in mice. They found that isolates of *C. albicans* with the highest phospholipase activity were also the most lethal.

The radiometric assay of the phospholipase activities in culture filtrates of three strains of *C. albicans* showed that all of these strains secreted both phospholipase B and lysophospholipase-transacylase. The egg yolk agar method of determining extracellular phospholipase activity likely detects phospholipase B. Although the radiometric measurements of lysophospholipase-transacylase activity also correlated with the total extracellular phospholipase activity of the organisms on egg yolk agar, this class of enzymes probably did not produce much of the precipitate seen on the egg yolk agar plates. This precipitate is composed of free fatty acids, and transacylase activity does not result in the liberation of fatty acids.

Each of the organisms tested secreted both phospholipase B and lysophospholipase-transacylase. Similarly, Takahashi et al. (25) found that five other strains of *C. albicans* produced both of these enzymes. Another study suggested that *C. albicans* produced both of these enzymes. Another study suggested that *C. albicans* secretes phospholipase A and phospholipase C (5). However, this conclusion was based on the isolation of palmitic acid and phosphorylcholine from the proximity of candidal colonies that were cultured on SDA supplemented with serum and sheep erythrocytes. Since this medium is not chemically defined, the sources of the hydrolysis products are uncertain.

The inability of other virulence factors to predict mortality does not preclude these factors from being important in the development of hematogenous candidal infections. Presumably, at least some of these factors were not predictive of mortality in our model because they exhibited less strain-to-strain variation than did extracellular phospholipase production. Alternatively, the expression of a virulence factor by a given isolate in vitro may not correlate with the expression of that factor in vivo.

Extracellular phospholipases are thought to contribute to virulence by lysing host cells or altering their surface characteristics such that adherence and penetration are facilitated. For example, it has been suggested that during infection by other organisms, such as *Rickettsia* spp. (24, 27, 29) and *T. gondii* (21), phospholipases may enhance adhesion to and cause lysis of host cell membranes. A direct apposition between *C. albicans* and epithelial cells has been observed in ultrastructural studies of epithelial invasion (18). It is possible that this fusion is facilitated by candidal phospholipases.

Barrett-Bee et al. (2) have found that yeasts with the highest extracellular phospholipase activity also adhered the most avidly to buccal epithelial cells. However, we found that there was no correlation between extracellular phospholipase production and endothelial cell adherence. This difference could have arisen because we measured the adherence of *C. albicans* to endothelial cells instead of epithelial cells. In addition, all of our isolates adhered avidly to the endothelial cells, so that any contribution of the candidal phospholipases to adherence may not have been evident.

Extracellular enzymes, including phospholipases and proteases, have been shown to damage host cell membranes (22). Previously, we have found that chemically induced mutants of *C. albicans* that were deficient in extracellular phospholipase activity caused ≥30% less damage to endothelial cells than did the parent strain (9). However, in the current study, we found no correlation between extracellular phospholipase or proteinase activity and endothelial cell injury. This lack of correlation suggests that other factors, which have not yet been elucidated, are also operative in endothelial cell injury by *C. albicans*.

The results of the present study strongly suggest that extracellular phospholipases secreted by *C. albicans* contribute to the virulence of this yeast. To answer more definitively whether extracellular phospholipases contribute to the pathogenesis of *C. albicans*, isogenic strain pairs that differ only in the production of extracellular phospholipase activity need to be constructed. The relative levels of virulence of these strains should then be compared in an animal model. This project is currently in progress.

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