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Adherence to and Damage of Endothelial Cells by *Cryptococcus neoformans* In Vitro: Role of the Capsule

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Escape from the intravascular compartment is likely a critical step in the development of hematogenously disseminated cryptococcal infections, such as meningitis. The capsule of *Cryptococcus neoformans* is considered to be a virulence factor because of its antiphagocytic properties. To further investigate the role of the capsule in escape from the intravascular compartment, we used isogenic strain pairs, an acapsular mutant, and an encapsulated clinical isolate to determine the effects of the capsule of *C. neoformans* on adherence to, phagocytosis by, and damage of endothelial cells in vitro. Acapsular *C. neoformans* adhered significantly more to endothelial cells and caused greater endothelial cell injury than did encapsulated organisms. Coating of an acapsular strain with cryptococcal glucuronoxylomannan decreased both adherence to and damage of endothelial cells by 61.7% ± 9.1% and 76.6% ± 10.2%, respectively. Transmission electron microscopy demonstrated internalization of acapsular, but not encapsulated, organisms by endothelial cells. Internalization of an acapsular strain occurred through endothelial cell phagocytosis and was inhibited by cytochalasin D. Phagocytosis required a heat-labile serum factor, probably complement. These results suggest that acapsular or poorly encapsulated *C. neoformans* may be the form(s) that escapes from the vasculature during initiation of hematogenously disseminated disease.

Infection with *Cryptococcus neoformans* is believed to be initiated by inhalation of airborne cells of the yeast from an environmental source (22, 30). From the alveoli, the organisms likely gain access to the bloodstream. Subsequent hematogenous dissemination causes deep-seated infection in target organs, such as the brain. Consequently, adherence to and passage through endothelial cells are likely to be early steps in the organism's escape from blood vessels and invasion of the target organ parenchyma.

Although the cryptococcal capsule is recognized as a major virulence factor (3–6), the capsule may be either small or absent during the initial phases of infection. Perfect (30) has suggested that only acapsular or sparsely encapsulated organisms are small enough to reach the alveoli via inhalation. Furthermore, it is known that organisms isolated from nature tend to be small and poorly encapsulated (1, 6, 29). Nevertheless, organisms isolated from cerebrospinal fluid are usually heavily encapsulated (22, 30). Thus, the organism likely changes the size of its capsule as it disseminates from the alveoli to other target organs. However, despite numerous studies demonstrating the importance of the capsule in the virulence of *C. neoformans* (2, 9, 18, 21), it is unknown at which step of infection the transition from sparse encapsulation to heavy encapsulation occurs.

To examine whether the degree of encapsulation influences the step of transmigration of the organism through the endothelial cell lining of the blood vessels, we examined the effects of the capsule on the ability of *C. neoformans* to adhere to and damage endothelial cells in vitro. In this study, we used one isogenic strain pair that differed only in the presence of a capsule, as well as an unrelated acapsular mutant and an encapsulated strain of *C. neoformans*.

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MATERIALS AND METHODS

Organisms and culture conditions. *C. neoformans* ATCC 36556 (encapsulated) and ATCC 52817 (acapsular mutant) were purchased from the American Type Culture Collection (Rockville, Md.). The organisms were maintained at 4°C on slants of Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, Mich.) and subcultured onto fresh medium every 3 to 4 weeks. After most of the experiments had been performed, an isogenic strain pair in which the *CAP59* gene was deleted became available. Thus, we confirmed key aspects of the effect of the capsule on the interactions of *C. neoformans* and endothelial cells by using this isogenic strain pair. It consisted of encapsulated parent strain B-4476 (*a ura5 CAP59 ADE2*) and its acapsular mutant TYCC33 (*a ura5 ΔCAP59 ADE2 ade2*). These organisms were generously provided by K. J. Kwon-Chung (National Institutes of Health, Bethesda, Md.). They were maintained at 4°C on slants of YEPD (1% yeast extract [Difco], 2% Bacto Peptone [Difco], 2% [wt/vol] glucose) supplemented with 20 μg each of uracil and adenine per ml.

The organisms were grown in yeast nitrogen base (YNB) with amino acids (Difco) enriched with 0.5% (wt/vol) glucose on a rotating drum at 27°C in room air for 72 h. When the isogenic strain pair was used, the YNB was supplemented with 20 μg each of uracil and adenine per ml. On the day of the experiment, the cells were harvested by centrifugation, washed twice in 0.85% (wt/vol) NaCl, and sonicated for 4 s (350 Sonifier; Branson Sonic Power Co., Danbury, Conn.). The yeast cells were counted in a hemacytometer and adjusted to the desired concentration in Hank's balanced salt solution (HBSS) with or without pooled human serum (PHS) (see below). Cell counts were confirmed by counting the number of CFU on SDA.

Preparation of serum. PHS was collected from healthy volunteers after informed consent was obtained. The blood samples were left at room temperature for 30 min to clot and then centrifuged at 600 × *g* for 10 min, and the serum was

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pooled and stored in aliquots at −70°C. To deplete the serum of complement activity, aliquots were heated at 56°C for 30 min.

Preparation of human endothelial cells. Human umbilical vein endothelial cells were obtained by a modification of the method of Jaffe et al. (17). The cells were harvested by using collagenase (Sigma) and were grown in M-199 (GIBCO, Grand Island, N.Y.) enriched with 10% fetal bovine serum (GIBCO), 10% defined bovine calf serum (Hyclone, Logan, Utah), L-glutamine, penicillin, and streptomycin. Second- or third-passage cells were grown to confluency in 24-well tissue culture plates (Costar, Van Nuys, Calif.) coated with a collagen matrix (Vitrogen; Celtrix, Palo Alto, Calif.). All incubations were in 5% CO_2 at 37°C.

Adherence of viable *C. neoformans* to endothelial cells. All adherence experiments were carried out in 24-well tissue culture plates by following a modification of our previously described method (10). Only confluent endothelial cells were used to avoid possible adherence of cryptococcal cells to plastic surfaces. Two methods were necessary to measure the adherence of live *C. neoformans* over a wide range of inoculum sizes. For small inocula, we used colony counting to directly determine the number of adherent organisms. At higher concentrations ($\geq 10^4$ organisms per well) inaccuracy due to clumping of the organisms and incomplete removal of the yeast from the wells was avoided by using a radiometric assay.

Measurement of adherence at low cell concentrations by colony counting. After the endothelial cell monolayers were rinsed twice with prewarmed HBSS, a 0.5-ml volume of a singlet blastospore suspension (3×10^2 cells) in HBSS, with or without 10% PHS, was added to each well. The inoculum size of each suspension was confirmed by culturing aliquots in SDA. The plate was incubated at 37°C for various time periods, after which the nonadherent organisms were aspirated and the endothelial cell monolayers were rinsed twice with 0.5 ml of HBSS in a standardized manner. One-half milliliter of SDA was added to each well and allowed to solidify. After the plate was incubated at 37°C for 48 h, the number of adherent organisms was determined by colony counting. All experiments were performed in replicates of at least three by using endothelial cells from different umbilical cords. Adherence was expressed as a percentage of the inoculum added (mean ± standard deviation [SD]).

Radiometric determination of adherence at high concentrations. To study adherence of *C. neoformans* to endothelial cells as a function of the yeast inoculum size (10^4 to 10^7 CFU per well), we labeled the yeast cells with L-[^{35}S]methionine (ICN, Irvine, Calif.) in accordance with a modification of our previously described method (26). Briefly, the yeast cells were grown in YNB supplemented with amino acids and 20 μg each of uracil and adenine per ml for 72 h. The cells were harvested, washed, and counted as described above. An inoculum of 2×10^8 organisms per 10 ml of YNB broth without amino acids (containing 70 μCi of L-[^{35}S]methionine) was incubated for 2 h at 30°C on a rotary shaker (250 rpm). The labeled cells were washed seven times with saline to remove the unincorporated radioactivity, counted, and resuspended in HBSS. The average specific activities of the acapsular and encapsulated organisms were 0.71 ± 0.04 and 0.23 ± 0.06 cpm per organism, respectively. After the endothelial cell monolayers were rinsed twice with prewarmed HBSS, 10^4 to 10^7 blastospores were added to each well in a total volume of 0.5 ml. The total number of organisms added to each well was determined by adding the same volume of labeled organisms directly to the scintillation cocktail. The plate was incubated at 37°C for 45 min. After incubation, the wells were aspirated and rinsed twice with 0.5 ml of HBSS to remove nonadherent yeast cells. The aspirates and rinses containing nonadherent organisms were combined for scintillation counting. For scintillation counting, all samples were placed in glass scintillation vials containing 20 ml of Ultima Gold scintillation cocktail (Packard, Meriden, Conn.) and the β emissions were measured in a Packard 2200CA liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). All experiments were performed in replicates of at least three. The number of adherent organisms was calculated by subtracting the number of nonadherent organisms from the total number of organisms added to the wells. We used this indirect method to determine the number of adherent organisms because we were unable to remove all of the adherent organisms from the wells, even with extensive rinsing. Similar problems have been encountered with *Candida albicans* at these concentrations (26).

Adherence of nonviable *C. neoformans* to endothelial cells. The effect of *C. neoformans* viability on adherence to endothelial cells was determined by direct visualization of the organisms. Yeast cells were killed by incubation in HBSS at 60°C for 30 min (33). An aliquot of the organisms was inoculated onto SDA to confirm that all of the cells had been killed. In parallel, control viable organisms were incubated in HBSS at 37°C. Aliquots of killed or viable organisms (1.5×10^5 cells) were incubated on endothelial cell monolayers in 24-well plates in quadruplicate and rinsed as previously described. At this concentration, clumping did not occur. The monolayers were fixed with methanol and stained with Giemsa, and adherent organisms were counted by light microscopy (31). At least 12 fields were counted, and the experiment was performed in triplicate with endothelial cells from different umbilical cords.

Endothelial cell damage by viable and nonviable *C. neoformans*. The ability of acapsular and encapsulated strains of *C. neoformans* to damage endothelial cells was determined by a modification of the ^{51}Cr release assay previously described (16). Endothelial cells grown in 24-well tissue culture plates were incubated with $Na_2{}^{51}CrO_4$ (ICN, Irvine, Calif.) in M-199 medium (2.5 μCi per well) for 16 h. On the day of the experiment, the unincorporated tracer was aspirated and the wells were washed three times with prewarmed HBSS. Endothelial cells were infected with 10^7 organisms per well in 1 ml of 10% (vol/vol) PHS in HBSS and incubated at 37°C for selected time intervals. Spontaneous ^{51}Cr release was determined by incubating endothelial cells in 10% PHS without organisms. At the end of the incubation period, 0.5 ml of medium was aspirated from each well and transferred to glass tubes for determination of ^{51}Cr activity. The wells were then treated with 6 N NaOH for 30 min and rinsed twice with 10% RadiacWash. The NaOH and 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 radioactive counts per min of the aspirated medium plus the radioactive counts of the endothelial cells lysed with NaOH and RadiacWash. After the data were corrected 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, and the experiment was performed in triplicate with endothelial cells from different umbilical cords.

To investigate whether endothelial cell damage requires direct contact between endothelial and yeast cells, organisms were added to cell culture membrane inserts (pore size, 0.45 μm; Falcon, Lincoln Park, N.J.) suspended above endothelial cells and specific ^{51}Cr release was determined as described above.

Binding of cryptococcal polysaccharide to the acapsular strain of *C. neoformans*. To investigate if the capsular polysaccharide plays a role in the adherence to and damage of endothelial cells, we used cryptococcal glucuronoxylomannan (GXM), the major constituent of the cryptococcal capsule (4), to form a capsule on the acapsular strain by a modification of the method of Kozel and Hermerath (19). Acapsular strain 52817 was grown in YNB and washed, and then 10^8 cells in 2 ml of phosphate-buffered saline (PBS) were incubated with 100 μg of polysaccharide per ml for 2 h at room temperature. The cells were washed three times with PBS, resuspended in 2 ml of HBSS, and used in adherence and damage experiments as described above. In a parallel experiment, to determine the effects of continuous exposure to GXM on adherence and endothelial cell injury, acapsular yeast cells were suspended in HBSS containing 100 μg of GXM per ml and then added to endothelial cells directly without removing the unbound GXM.

Quantitation of GXM bound to the acapsular strain of *C. neoformans*. Binding of cryptococcal GXM to acapsular *C. neoformans* 52817 was confirmed by using radiolabeled GXM. The cryptococcal GXM was coupled with tyramine and then labeled with ^{125}I as described previously (8, 19). The ^{125}I-labeled GXM was mixed with unlabeled GXM to produce a specific activity of 2.9×10^4 cpm per μg of GXM. Two hundred micrograms of the labeled GXM was added to 10^8 cells of *C. neoformans* 52817 in 2 ml of PBS, after which the organisms were incubated for 2 h at room temperature. The cells were washed three times with PBS, and the cell-associated radioactivity was measured with a scintillation counter to determine the amount of GXM bound. The experiment was done in quadruplicate, and the data were expressed as nanograms of bound GXM per 10^6 organisms.

Transmission electron microscopy. Confluent endothelial cell monolayers were incubated at 37°C for 5 h with a 1-ml volume of HBSS containing 5×10^6 cells of *C. neoformans* and 10% PHS. After incubation, the monolayers were washed twice with prewarmed HBSS, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Sigma), and washed three times in the same buffer. Samples were processed by the method of Haudenschild et al. (14) and examined in a Hitachi HU-125-E electron microscope (Tokyo, Japan).

Assay for phagocytosis of *C. neoformans* by endothelial cells. To quantify endothelial cell internalization of *C. neoformans*, we used a modification of the method of Levitz et al. (24). Confluent endothelial cells grown on glass coverslips were infected with 10^5 acapsular or encapsulated *C. neoformans* cells in the presence of either 10% PHS or 10% heat-inactivated PHS. After 5 h, the non-cell-associated yeast cells were aspirated and the monolayers were rinsed once with warm HBSS. The cells were then fixed in 2% glutaraldehyde, and the *C. neoformans* cells were stained with 1% Uvitex RSB (a kind gift from Jay Isharani, Ciba-Geigy, Greensboro, N.C.) for 1 h. Each coverslip was inverted onto a slide with 1 drop of glycerol and sealed with nail polish. The total number of cell-associated organisms was determined by phase-contrast microscopy. The same field was examined by epifluorescence microscopy, and the number of uninternalized yeast cells (which were brightly fluorescent) was determined. The number of internalized organisms was calculated by subtracting the number of fluorescent organisms from the total number of visible organisms. At least 100 organisms were counted in five different fields. Each experiment was performed in duplicate.

Determination of the relationship between phagocytosis of *C. neoformans* and endothelial cell injury. To investigate if endothelial cell injury requires internalization of *C. neoformans*, we studied the effects of disruption of endothelial cell microfilaments with cytochalasin D (Sigma) on cryptococcal internalization by and damage of endothelial cells. Selected concentrations of cytochalasin D were added to endothelial cells simultaneously with *C. neoformans*, and endothelial cell injury and internalization of the organisms were determined as described above. In damage experiments, control wells containing cytochalasin D without organisms were included to evaluate the toxicity of cytochalasin D. The highest concentration of cytochalasin D used (0.1 μM) caused less than 5% specific release of ^{51}Cr in all experiments. Each experimental condition was tested in

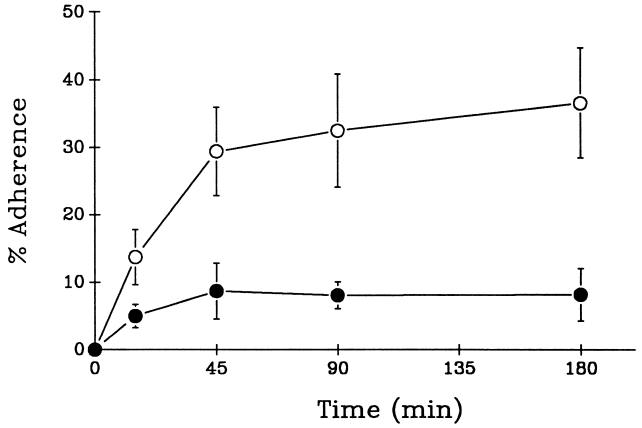

FIG. 1. Time course of adherence to endothelial cells by acapsular (○) and encapsulated (●) strains of *C. neoformans* (3×10^2 cells per well). Adherence to endothelial cells was evaluated by counting the adhering CFU of the yeast (n = 10 determinations). Error bars, SD.

replicates of three, and the experiment was performed in triplicate with endothelial cells from different umbilical cords.

Growth studies. The effect of cytochalasin D on the growth rate of acapsular *C. neoformans* 52817 was investigated. Yeast cells were grown in YNB, washed, and counted as described above. These cells were used to inoculate 50 ml of Sabouraud dextrose broth with or without 0.1 μM cytochalasin D at an initial concentration of 10^6 organisms per ml. The organisms were incubated at 37°C on a rotary shaker. At intervals, 1 ml of each suspension was withdrawn and mixed well and the A_{420} was determined spectrophotometrically.

Statistical analysis. The results of similar experiments performed on different days were compared by the Mann-Whitney U test for single comparisons. When multiple experimental conditions were compared, the Kruskal-Wallis test with the Tukey correction for multiple comparisons was used. P values of ≤ 0.05 were considered significant.

RESULTS

Cryptococcal capsule prevents adherence to, internalization by, and damage of endothelial cells in vitro. As shown in Fig. 1, both acapsular (strain 52817) and encapsulated (strain 36556) *C. neoformans* cells adhered to endothelial cells as early as 15 min after addition of the yeast. Adherence increased with time and reached a plateau after 45 min. Therefore, an incubation time of 45 min was chosen for all subsequent adherence experiments. The acapsular strain adhered to endothelial cell monolayers significantly more than did the encapsulated strain at all of the time points tested ($P < 0.001$ at all time points). We found also that the acapsular strain was internalized by endothelial cells significantly more than was the encapsulated strain. By the differential fluorescence assay, we determined that 77.1% ± 6.9% of acapsular cells were internalized, compared with 8.0% ± 3.3% of encapsulated *C. neoformans* cells ($P < 0.001$). Internalization of the acapsular strain was confirmed by transmission electron microscopy (Fig. 2).

As in the adherence and internalization experiments, at 10^7 organisms per well, acapsular strain 52817 caused significantly

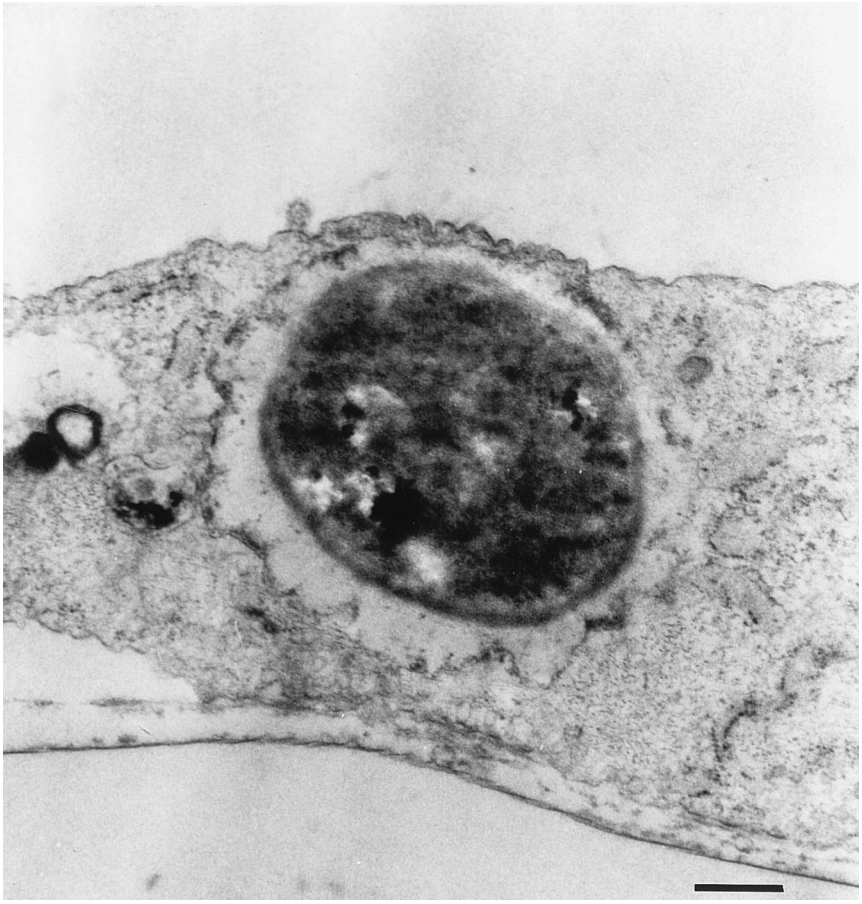

FIG. 2. Transmission electron micrograph of endothelial cells infected with acapsular *C. neoformans*. Magnification, ×15,000; bar, 1 μm.

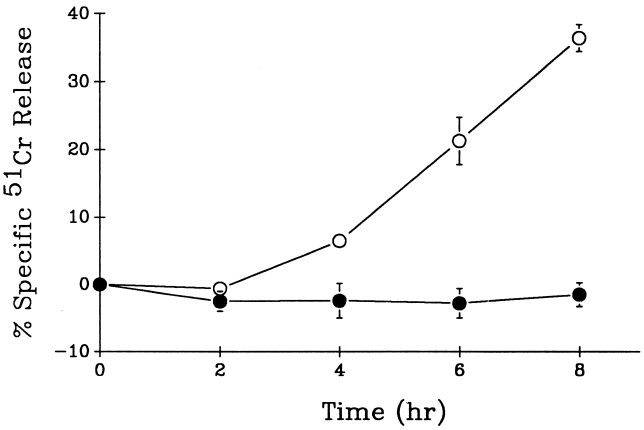

FIG. 3. Time course of damage of endothelial cells by acapsular (○) and encapsulated (●) strains of *C. neoformans* (10^7 cells per well). Damage of endothelial cells was measured by ^{51}Cr release ($n = 6$ determinations). Error bars, SD.

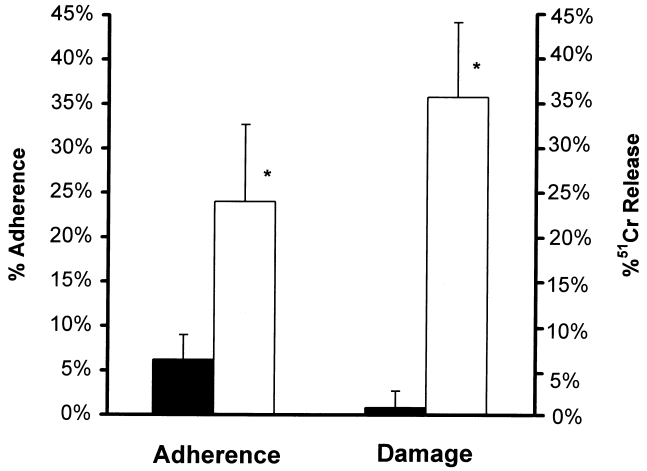

FIG. 4. Adherence to and damage of endothelial cells caused by the isogenic strain pair. Strain B-4476 is encapsulated (■), and strain TYCC33 is acapsular (□) ($n = 18$ determinations). *C. neoformans* cells (3×10^2) were incubated with endothelial cells at 37°C for 45 min in the adherence assay and for 8 h in the damage assay. *, $P < 0.001$. Error bars, SD.

greater endothelial cell damage than did encapsulated strain 36556 (Fig. 3). Damage of endothelial cells by the acapsular strain was detectable as early as 4 h and increased in a time-dependent manner, reaching 36.4% ± 2.0% after 8 h. Therefore, an incubation time of 8 h was chosen for all subsequent damage experiments. In contrast, the encapsulated strain caused no detectable endothelial cell injury at any time point. Preliminary damage experiments with different inocula of acapsular *C. neoformans* showed that an inoculum of 2.5×10^6 organisms per well was required to cause detectable endothelial cell injury (data not shown).

Confirmation of the role of the capsule in adherence to and damage of endothelial cells with an isogenic strain pair. After the experiments comparing strains 52817 and 36556 were completed, an isogenic pair of strains which differ in the presence of a single capsule gene became available (from K. J. Kwon-Chung). We used this strain pair to test further whether these differences in adherence and damage are due to the presence of the capsule. The adherence of acapsular deletion mutant strain TYCC33 was 3.9-fold higher than that of encapsulated parent strain B-4476 (Fig. 4). Similarly, the acapsular mutant caused significant endothelial cell injury, whereas the encapsulated parent did not (Fig. 4).

We examined the possibility that the differences in internalization and damage caused by the two isogenic organisms were due to differences in their adherence. We tested the adherence of the organisms with larger inocula, such as those used in the internalization and damage experiments. With inocula of 10^4 to 10^6 organisms per well, the acapsular deletion mutant still adhered significantly more than the encapsulated parent strain ($P < 0.001$) (Fig. 5). However, at 10^7 organisms per well (the inoculum size used in the damage experiments), there was no significant difference in the adherence of either strain to endothelial cells ($P > 0.5$).

Binding of cryptococcal GXM to the acapsular *C. neoformans* strain decreases its adherence to, internalization by, and damage of endothelial cells. After exposure of acapsular strain 52817 to exogenous GXM, 71 ± 1.3 ng of GXM was bound per 10^7 organisms. Assuming a polysaccharide molecular weight of 500,000, approximately 85,000 molecules of GXM per yeast cell were bound under these conditions.

Precoating of the acapsular strain with GXM decreased its adherence to endothelial cells by 61.7% ± 9.1% as determined by counting the CFU ($P < 0.001$) (Table 1). By transmission electron microscopy, we determined that formation of a capsule on the acapsular strain by coating it with GXM also inhibited the internalization of this organism. Finally, GXM coating inhibited *Cryptococcus*-induced endothelial cell damage by 76.6% ± 10.2% as determined by the ^{51}Cr release assay ($P < 0.001$) (Table 1). Furthermore, simultaneous addition of GXM and the acapsular strain to endothelial cells resulted in inhibition of both adherence to and damage of endothelial cells by the acapsular strain. At a concentration of 100 μg of GXM per ml, adherence was decreased by 69.5% ± 18.0% compared with the control ($P < 0.001$) and endothelial cell injury was diminished by 83.9% ± 10.5% ($P < 0.001$) (Table 1). Addition of GXM alone did not affect ^{51}Cr release. The inhibition of adherence to and damage of endothelial cells obtained when both the organisms and the endothelial cells were exposed to GXM was not significantly different from the inhibition obtained when precoated acapsular cells were used.

Adherence to and damage of endothelial cells by acapsular organisms depend upon cryptococcal viability, but internalization does not. Killed acapsular organisms (strain 52817) had a

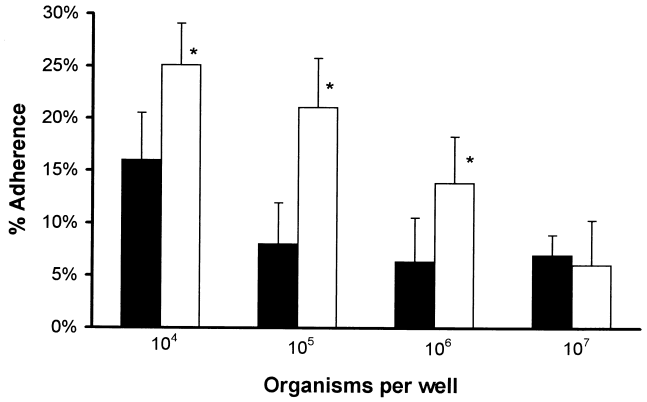

FIG. 5. Effect of yeast inoculum size on adherence of the isogenic strain pair to endothelial cells. Strain B-4476 is encapsulated (■), and strain TYCC33 is acapsular (□) ($n = 9$ determinations). *C. neoformans* cells were incubated with endothelial cells at 37°C for 45 min, and percent adherence was calculated by counting the ^{35}S radioactivity. *, $P < 0.001$. Error bars, SD.

TABLE 1. Effect of coating acapsular *C. neoformans* with cryptococcal GXM on adherence to and damage of endothelial cells

Treatment	% Adherence[a]	% Specific ^{51}Cr release[b]
Control	25.6 ± 5.2	23.1 ± 4.2
GXM pretreatment[c]	9.6 ± 2.6[d]	7.2 ± 3.6[d]
GXM in assay[e]	7.7 ± 5.0[d]	3.4 ± 2.1[d]

[a] Organisms (3×10^2) were incubated with endothelial cells for 45 min.
[b] Organisms (10^7) were incubated with endothelial cells for 8 h.
[c] Acapsular cells (10^8) were incubated with 100 μg of GXM per ml in PBS for 2 h and then washed with PBS before use in the adherence or damage assay ($n = 9$ determinations).
[d] $P < 0.001$ compared with the control.
[e] GXM (100 μg/ml) was introduced directly in the adherence or damage assay ($n = 9$ determinations).

62.5% ± 12.0% reduction in adherence to endothelial cells compared with live organisms ($P < 0.001$). Viable and nonviable encapsulated organisms (strain 36556) did not significantly differ in adherence (data not shown), probably because of the low adherence of encapsulated organisms to endothelial cells.

Killing of acapsular *C. neoformans* had disparate effects on internalization and endothelial cell injury. Killed acapsular organisms were internalized to the same extent as viable organisms. In contrast, dead acapsular organisms caused 63.9% ± 7.0% less endothelial cell injury than did viable yeast cells ($P < 0.001$).

Extent of endothelial cell injury correlates with amount of internalization of acapsular *C. neoformans*. We first determined whether contact with endothelial cells is required for the organisms to cause injury. When acapsular organisms (strain 52817) were separated from endothelial cells by cell culture membrane inserts, there was no detectable endothelial cell injury (data not shown).

We next examined whether internalization of the organisms is required for the acapsular strain to cause endothelial cell injury. When selected concentrations of cytochalasin D were added to endothelial cells simultaneously with acapsular *C. neoformans*, internalization of the organisms was reduced by up to 81.9% ± 1.6% ($P < 0.001$). This reduction in internalization was accompanied by a 77.6% ± 8.5% decrease in endothelial cell injury ($P < 0.001$). After 8 h of exposure, cytochalasin D had no effect on the growth of *C. neoformans* as determined by spectrophotometric assay and colony counting (data not shown).

Acapsular cryptococcal internalization and damage of endothelial cells depend on a heat-labile serum factor(s). The presence of PHS had no effect on the adherence of either acapsular (strain 52817) or encapsulated (strain 36556) organisms to endothelial cells. The adherence of the acapsular strain was 24.8% ± 6.2% in the presence of 10% PHS and 24.0% ± 9.8% in HBSS alone. Similarly, the encapsulated strain exhibited 9.5% ± 4.1% and 8.9% ± 5.4% adherence in the presence and absence of PHS, respectively.

In contrast, endothelial cell internalization of acapsular *C. neoformans* required a heat-labile serum opsonin, since internalization was decreased from 77.1% ± 6.9% to 15.2% ± 5.5% when the organisms were added to endothelial cells in heat-inactivated PHS ($P < 0.001$). Similarly, endothelial cell damage caused by acapsular *C. neoformans* was reduced from 28.4% ± 8.0% to 1.5% ± 3.2% when heat-inactivated PHS was used instead of normal PHS ($P < 0.001$). Incubation of endothelial cells with HBSS containing 10% heat-inactivated PHS did not alter the spontaneous release of ^{51}Cr (data not shown).

DISCUSSION

The mechanism by which *C. neoformans* egresses from the vascular compartment is not fully understood. Our hypothesis is that blood-borne organisms adhere to endothelial cells and then are phagocytized by these cells. Escape of these ingested organisms into the tissue parenchyma may occur by injury and death of the endothelial cells. An alternative hypothesis is that the organisms are carried from the bloodstream into the tissue parenchyma by circulating phagocytes. However, these ingested organisms would likely be killed by the phagocytes (5, 25).

Endothelial cells have been found to ingest a variety of microbial pathogens. We have found that human umbilical vein endothelial cells phagocytize *C. albicans* (7, 31). Recently, endothelial cell phagocytosis of *Histoplasma capsulatum* has also been reported (15). Furthermore, studies on the interactions between bacteria and endothelial cells have shown that group B streptococci (11), *Rickettsia prowazekii* (36), *Haemophilus influenzae* (35), and *Staphylococcus aureus* (13) are phagocytized by endothelial cells.

Since environmental isolates of *C. neoformans*, which are the likely source of human infections, are either acapsular or sparsely encapsulated, and since the capsule is known to affect adherence to and internalization of the organism by a variety of host cells (18, 20, 27), we examined the effects of the capsule on the interaction of *C. neoformans* with endothelial cells. By using both an isogenic strain pair and two unrelated organisms, we found that acapsular organisms adhered better, were internalized more, and caused greater damage to endothelial cells than did encapsulated organisms. The presence of a capsule has been shown to inhibit the interaction of other important microbial pathogens with endothelial cells. For example, acapsular mutants of *H. influenzae* have higher adherence and are internalized more readily by human umbilical vein endothelial cells than are encapsulated organisms (35). Similarly, type III capsular polysaccharide attenuates the phagocytosis of group B streptococci by human umbilical vein endothelial cells (11).

The acapsular organisms exhibited higher endothelial cell adherence than did encapsulated organisms at all inoculum sizes of less than 10^7 organisms per well. Similarly, Merkel and Scofield (27) reported that acapsular *C. neoformans* organisms adhere more avidly to glial cells than do encapsulated organisms. Therefore, acapsular organisms likely have adhesins that differ from those of encapsulated organisms or the capsule interferes with the function or expression of at least some of these adhesins. Merkel and Cunningham (28) proposed that the cryptococcal adhesin mediating binding to epithelial cells is either a protein or a glycoprotein. They found adherence to be significantly decreased after the organisms were exposed to trypsin. As in our studies, they found that the adherence of encapsulated organisms was not affected by heat killing of the organisms. These results suggest that the adhesin on encapsulated organisms is heat stable. We observed that in contrast to that of encapsulated organisms, adherence of the acapsular strain was decreased by 62.5% ± 12.0% when the organisms were heat killed. This finding is evidence that at least one of the adhesins on this form of the organism is heat labile.

As the number of organisms added to the endothelial cells was increased, the percentage of adherent organisms decreased for both acapsular and encapsulated organisms. In addition, the difference in adherence between the acapsular and encapsulated *C. neoformans* strains decreased with increasing inoculum size. When 10^7 *C. neoformans* cells were added to each well of endothelial cells, there was no difference in adherence between these two organisms. Both of these phe-

nomena may be explained by nonspecific binding and/or saturation of the host receptors at high yeast cell concentrations.

Transmission electron microscopy and the fluorescent internalization assay showed that the presence of a capsule decreased ingestion of the yeast by endothelial cells. Merkel and Cunningham (28) found similarly that pulmonary epithelial cells are able to internalize both viable and formalin-killed encapsulated *C. neoformans* cells in vitro. Those investigators did not examine the internalization of acapsular organisms (28). In our study, inhibition of endothelial cell internalization by cytochalasin D indicated that this internalization occurs by the process of phagocytosis.

Unlike that of *C. albicans* (7), phagocytosis of acapsular *C. neoformans* by endothelial cells requires the presence of a heat-labile serum factor. Therefore, it is likely that adherence and phagocytosis of the organisms are mediated by different receptors. The serum factor required for internalization is likely one of the components of the classical complement pathway. Acapsular organisms activate both the classical and the alternative pathways, while encapsulated organisms activate only the alternative pathway (37). One potential candidate opsonin is C1q, which has been shown to mediate the phagocytosis of *Salmonella minnesota* by endothelial cells (32). Whether C1q also mediates the phagocytosis of *C. neoformans* remains to be determined. In addition, to our knowledge, the presence of receptors for C1q on endothelial cells has not been directly determined.

Internalization of *C. neoformans* was required for endothelial cell injury to occur. All conditions that were associated with decreased internalization of the acapsular organism also reduced endothelial cell injury. These conditions included the presence of GXM, the use of heat-inactivated PHS, and exposure of endothelial cells to cytochalasin D. Endothelial cell injury is due, in part, to a heat-labile factor, since heat-killed organisms were internalized but caused significantly less endothelial cell injury than did viable cells. *C. neoformans* is known to secrete DNase (3) and proteinase (34). Therefore, it is possible that these enzymes contribute to endothelial cell injury. However, since killing of the organisms did not block endothelial cell injury completely, either phagocytosis per se or some factor intrinsic to the cryptococcal cell wall also damages endothelial cells.

At a concentration of 10^7 organisms per well, the acapsular mutant caused significantly more endothelial cell injury than did the encapsulated parent, but these two strains did not differ in adherence at this high concentration. Therefore, cryptococcal adherence and endothelial cell damage are distinct processes.

Transformation from the acapsular or sparsely encapsulated to the heavily encapsulated state is of critical importance in the development and maintenance of disseminated cryptococcal infections. Although organisms with large capsules may have a survival advantage due to their ability to avoid host defense mechanisms in established infections, the smaller acapsular organisms may be the form that initiates cryptococcal infections. The latter form of the organism is better able to reach the alveoli. Interestingly, by using a rat model, Goldman et al. showed that *C. neoformans* seems to shed its capsule shortly after deposition in the alveoli (12). Moreover, acapsular strains of *C. neoformans* are more resistant to killing by murine bronchoalveolar macrophages than are encapsulated organisms (23). The impaired ability of bronchoalveolar macrophages to kill acapsular *C. neoformans* may give this form of the organism the advantage in initiating an infection. Finally, acapsular or sparsely encapsulated organisms may be more efficient at disseminating hematogenously, since we have found that such organisms avidly adhere to and are phagocytized by endothelial cells. Our results suggest that both the acapsular and encapsulated forms of the organism should be the focus of studies of mechanisms of pathogenicity and the development of targeted therapeutic modalities.

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