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Penetration and Damage of Endothelial Cells by *Candida albicans*

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The mechanisms of phagocytosis of *Candida albicans* by human vascular endothelial cells and subsequent endothelial cell injury were examined in vitro. Both live and killed *C. albicans* cells were phagocytized by endothelial cells. This organism specifically induced endothelial cell phagocytosis because neither *Candida tropicalis* nor *Torulopsis glabrata* was ingested. Endothelial cell microfilaments polymerized around *C. albicans* as the organisms were phagocytized. Cytochalasin D inhibited this polymerization of microfilaments around *C. albicans* and blocked phagocytosis. The blocking of actin depolymerization with phalloidin had no effect on microfilament condensation around the organism, indicating that the microfilaments surrounding *C. albicans* are formed from a pool of G-actin. Intact microtubules were also necessary for the phagocytosis of *C. albicans*, since the depolymerizing of endothelial cell microtubules with nocodazole prevented the condensation of actin filaments around the organisms and inhibited phagocytosis. In contrast, microtubule depolymerization was not required for microfilament function because the blocking of microtubule depolymerization with taxol had no effect on microfilament condensation around *C. albicans*. The phagocytosis of *C. albicans* was pivotal in the induction of endothelial cell damage, since the blocking of candidal internalization significantly reduced endothelial cell injury. Endothelial cells were not damaged by phagocytosis of dead organisms, indicating that injury was caused by a factor associated with viable organisms. Therefore, *C. albicans* is uniquely able to induce endothelial cell phagocytosis by comparison with non-*albicans* species of *Candida*. Furthermore, at least two components of the endothelial cytoskeleton, microfilaments and microtubules, are necessary for the phagocytosis of *C. albicans*.

In certain compromised hosts, *Candida albicans* disseminates hematogenously and causes widespread microabscesses throughout the body. Vascular endothelium plays a critical role during the initiation of hematogenous infections, since bloodborne organisms likely adhere to and penetrate through the endothelial cell lining of the blood vessels to gain access to the tissue parenchyma. Thus, blocking the ability of *C. albicans* to escape from the intravascular compartment is a potential method to enhance the host defense against this organism and prevent the development of disseminated candidal infections.

Considerable effort has been devoted to characterizing the receptors and ligands involved in the adherence of *C. albicans* to endothelial cells and subendothelial cell matrix components (16, 25). However, less is known about the events that occur after the organism has adhered to endothelial cells. Rotrosen et al. (31) discovered that endothelial cell penetration by *C. albicans* in vitro occurred via phagocytosis of the organism by endothelial cells. We have found that contact with *C. albicans*, but not with *Candida tropicalis* and *Torulopsis glabrata*, induces two endothelial cell responses: endothelial cell injury and the synthesis and release of prostaglandins (10). Although these two processes occur in parallel, they are regulated independently (9). For example, stimulation of prostaglandin production is independent of protein kinase C, while the induction of endothelial cell damage is at least partially dependent on this class of enzymes. Furthermore, the blocking of prostaglandin production does not alter the extent of *Candida*-induced endothelial cell injury, indicating that the synthesis of prostaglandins does not modulate endothelial cell damage.

Recently, it has been determined that at least 10 important microbial pathogens may invade the host by inducing their own phagocytosis by nonprofessional phagocytic host cells, such as epithelial and endothelial cells. For example, *Yersinia pseudotuberculosis* expresses invasin, which binds to multiple β1 integrins on epithelial cells and stimulates the phagocytosis of this organism (22, 23). *Salmonella typhimurium* induces its own internalization by a different mechanism, which may involve activation of epidermal growth factor and 5-lipoxygenase (12, 29). Finally, *C. albicans* is known to be phagocytized by epidermal cells (5), as well as by endothelial cells (31). While we have shown that *C. albicans* adheres to endothelial cells and stimulates certain endothelial cell responses, the mechanisms by which endothelial cells phagocytize *C. albicans*, or other microbial pathogens, has not been examined in depth.

In the present study, to elucidate the mechanisms by which *C. albicans* is phagocytized by and injures endothelial cells, we examined the roles of endothelial cell microfilaments and microtubules in the phagocytosis of the organism and the subsequent development of endothelial cell injury.

MATERIALS AND METHODS

Inhibitors of cytoskeletal function. Cytochalasin D (Sigma Chemical Company, St. Louis, Mo.), nocodazole [methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate; Sigma], and taxol (Calbiochem, San Diego, Calif.) were dissolved in dimethyl sulfoxide and stored as stock solutions (at 1.97 mM, 33.2 mM, and 5.86 mM, respectively) at −70°C. Before they were added to the endothelial cells, these inhibitors were diluted in Hanks balanced salt solution (HBSS) so that the final concentration of dimethyl sulfoxide was less than 0.05% (vol/vol). In all experiments, control endothelial cells were incubated in the same

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concentration of dimethyl sulfoxide without the inhibitor. Colchicine and phalloidin (both from Sigma) were dissolved in HBSS on the day of the experiment.

Organisms. *C. albicans* ATCC 36082 (a clinical isolate) was obtained from the American Type Culture Collection (Rockville, Md.). *C. tropicalis* 4265 and *T. glabrata* 609 were clinical isolates obtained from the microbiology laboratory at Harbor-UCLA Medical Center. Growth and harvesting of the organisms were done as described previously (10). Briefly, the organisms were grown overnight in yeast nitrogen base broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.15% (wt/vol) L-asparagine and 2% (wt/vol) glucose on a rotating drum at 27°C. They were harvested by centrifugation, washed twice in 0.85% saline, and sonicated for 3 s to yield a suspension of singlet blastospores. The organisms were counted in a hemacytometer and adjusted to the desired concentration with either HBSS or RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.), depending on the experiment.

To obtain germinated organisms, the blastospores were suspended in RPMI 1640 medium at 3×10^6 organisms per ml and incubated on a rotary shaker at 37°C for 90 min. More than 90% of the organisms produced germ tubes when grown under these conditions. The killing of either the blastospores or germ tubes was accomplished by incubating them in 20 mM sodium periodate for 30 min at room temperature (31). The dead organisms were extensively washed in 0.85% saline prior to their addition to the endothelial cells (see below). Killing was confirmed by the plating of an aliquot of the organisms on Sabouraud dextrose agar (Difco).

Endothelial cells. Endothelial cells were isolated from human umbilical veins by the method of Jaffe et al. (24). The cells were grown in M-199 (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Intergen, Purchase, N.Y.), 10% defined bovine calf serum (Hyclone, Logan, Utah), and 2 mM L-glutamine, with penicillin and streptomycin (Irvine Scientific). Second- or third-passage endothelial cells were grown on a collagen matrix (Vitrogen; Celtrix, Palo Alto, Calif.) either in 24-well tissue culture plates (Falcon, Lincoln Park, N.J.) or on circular glass coverslips (diameter, 12 mm).

Endothelial cell damage. The amount of endothelial cell injury caused by *C. albicans* was quantified by a modification of our previously described chromium release assay (10). This assay reflects endothelial cell injury as determined by morphologic assessment and inhibition of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (8). Briefly, confluent endothelial cells in 24-well plates were incubated overnight in M-199 containing $Na_2{}^{51}CrO_4$ (6 μCi per well; ICN Biomedicals, Irvine, Calif.). The next day, the unincorporated tracer was aspirated and the wells were rinsed three times with warm HBSS. One milliliter of HBSS containing 10^6 *C. albicans* cells was added to each well, and the plate was incubated for 3 h at 37°C in 5% CO_2. This incubation period was chosen because, in preliminary experiments, we determined that significant *Candida*-induced endothelial cell injury occurred by 3 h while toxicity due to the inhibitors of cytoskeletal function remained low. At the end of the incubation, 0.5 ml of medium was gently aspirated from each well, after which the endothelial cells were lysed by the addition of 0.5 ml of 6 N NaOH. The lysed cells were aspirated, and the wells were rinsed twice with RadicWash (Atomic Products, Inc., Shirley, N.Y.). These rinses were added to the lysed cells, and the ^{51}Cr activity of the medium and the cell lysates was determined. Control wells containing HBSS but no organisms were processed in parallel to measure the spontaneous release of ^{51}Cr. After corrections were made for the differences in the incorporation of ^{51}Cr in each well, the specific release of ^{51}Cr was calculated by the following formula: $(2 \times \text{experimental release} - 2 \times \text{spontaneous release})/(\text{total incorporation} - 2 \times \text{spontaneous release})$.

In experiments designed to determine the effects of the inhibitors of cytoskeletal function on *Candida*-induced endothelial cell injury, the endothelial cells were incubated with 0.5 ml of a given inhibitor for 20 min and then 10^6 *C. albicans* cells suspended in 0.5 ml of the inhibitor were added to each well. The endothelial cell toxicity of the inhibitors of cytoskeletal function was monitored in wells containing endothelial cells and the pharmacologic agent, without organisms. At the concentrations used, all inhibitors caused a less than 5% specific release of ^{51}Cr. In other experiments, to determine if *C. albicans* released a soluble substance that caused endothelial cell injury, the organisms were grown in cell culture inserts (pore size, 0.45 μm; Falcon) suspended approximately 1 mm above the endothelial cells. After a 3-h incubation, the amount of ^{51}Cr released by the endothelial cells into the medium was determined as described above. All experiments were performed in triplicate.

Germ tube elongation. To determine if the inhibitors of cytoskeletal function affected *C. albicans*, elongation of candidal germ tubes in the presence and absence of the inhibitors was measured as described previously (21). Briefly, the endothelial cells were exposed to either HBSS or the various inhibitors for 20 min as described above, and 5×10^4 organisms, suspended in either HBSS or the maximal concentration of each inhibitor used in the chromium release experiments, were added to the endothelial cells. At selected time intervals, the medium was aspirated from the wells and 0.5 ml of 2% (vol/vol) glutaraldehyde in phosphate-buffered saline (PBS) was added. Next, the wells were examined with an inverted microscope and the average length of 100 germ tubes per condition was measured with a micrometer.

Fluorescent microscopy. The endothelial cell microfilaments were visualized with BODIPY 581/591-phalloidin (Molecular Probes, Eugene, Oreg.) by using a modification of the manufacturer's instructions. Endothelial cells grown on coverslips were incubated for selected times with 2×10^5 *C. albicans* cells. When an inhibitor of cytoskeletal function was used, the endothelial cells were incubated with the inhibitor for 20 min prior to the addition of the organisms and the inhibitor was present in the medium until the cells were fixed. In experiments designed to examine the phagocytosis of *C. tropicalis* or *T. glabrata*, the endothelial cells were incubated with 10^6 organisms for 2 h. At the end of the incubation period, the endothelial cells were fixed with 3.7% (vol/vol) formaldehyde in PBS and permeabilized in acetone. The microfilaments were stained by inverting the coverslips onto 50 μl of 2.5% (vol/vol) BODIPY-phalloidin in PBS for 20 min in a humidified chamber and then by rinsing in PBS. To visualize the *C. albicans* cells, the coverslips were inverted onto 50 μl of 1% (vol/vol) uvitex 2B (a generous gift from Jay Isharani, Ciba-Geigy, Greensboro, N.C.) (26) in PBS for 30 min and rinsed in PBS.

To visualize the endothelial cell microtubules, the cells were rinsed in PBS and incubated with 0.2% Triton X-100 and 3.7% formaldehyde in microtubule stabilization buffer [100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 2 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (pH 6.8)] (38) for 5 min at room temperature. Next, they were fixed in ice-cold methanol for 5 min and rehydrated in PBS for 45 min. The coverslips were incubated first with a murine anti-α-tubulin monoclonal antibody (Sigma) and then with fluorescein-labelled goat anti-mouse antibodies (Sigma). The *C. albicans* cells were visualized by light microscopy in these experiments instead of with the uvitex, because the uvitex caused depolymerization of the microtubules.

In all studies, the stained coverslips were mounted on slides with nail polish and SlowFade (Molecular Probes). The endothelial cytoskeletons were examined with either a Zeiss Axiovert 10 microscope (Carl Zeiss, Inc., Thornwood, N.Y.) equipped for epifluorescence microscopy or a Bio-Rad MRC 600 confocal microscope (Bio-Rad Laboratories, Hercules, Calif.). The cells were photographed with Ektachrome 400 film (Eastman Kodak, Rochester, N.Y.).

Electron microscopy. Transmission electron microscopy of endothelial cells infected with *C. albicans* was performed as previously described (31). Briefly, endothelial cells were grown to confluency in 35-mm-diameter petri dishes. They were incubated with the inhibitors as described above, and 5×10^6 live or periodate-killed organisms were added to each dish. After incubation at 37°C in 5% CO_2 for 2 h, the cells were rinsed once with HBSS and fixed with 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer. Next, the specimens were processed by the method of Haudenschild et al. (19) and examined with a Hitachi HU-125-E electron microscope (Hitachi, Tokyo, Japan).

RESULTS

Endothelial cells surround *C. albicans* with microfilaments during phagocytosis. When live blastospores of *C. albicans* were added to the endothelial cells, the organisms began germinating after 30 min, and virtually all organisms had germinated by 1 h. The germ tubes elongated progressively during continued exposure to the endothelial cells. Staining of the filamentous actin of the endothelial cells with BODIPY-phalloidin revealed that tubes of microfilaments formed around the germinated organisms (Fig. 1). These tubes were composed solely of microfilaments from endothelial cells because the BODIPY-phalloidin did not label the microfilaments of *C. albicans* (i.e., in Fig. 1A and E, the organisms that were not inside the endothelial cells were not stained). The polymerization of endothelial cell microfilaments around the organisms was maximal at 2 h and then appeared to regress, so the organisms that were already internalized no longer had actin filaments condensed around them. This phenomenon was observed both in time course experiments and by confocal microscopy (data not shown). It appeared that endothelial cell microfilaments polymerized only around *C. albicans* cells that were being actively phagocytized.

At all time points, the germinated organisms were only partially ingested by the endothelial cells (Fig. 1A and B). Usually, the actin polymerized around the germ tube portion of the organism, but occasionally polymerization of actin around the blastospore portion of the organism occurred. The condensation of endothelial cell microfilaments was not dependent on the viability of the organisms, since tubes of actin formed around periodate-killed organisms (Fig. 1C and D). Killed blastospores of *C. albicans* also stimulated the polymerization of actin (Fig. 1E and F).

By transmission electron microscopy, we confirmed that the polymerization of actin was accompanied by the endothelial

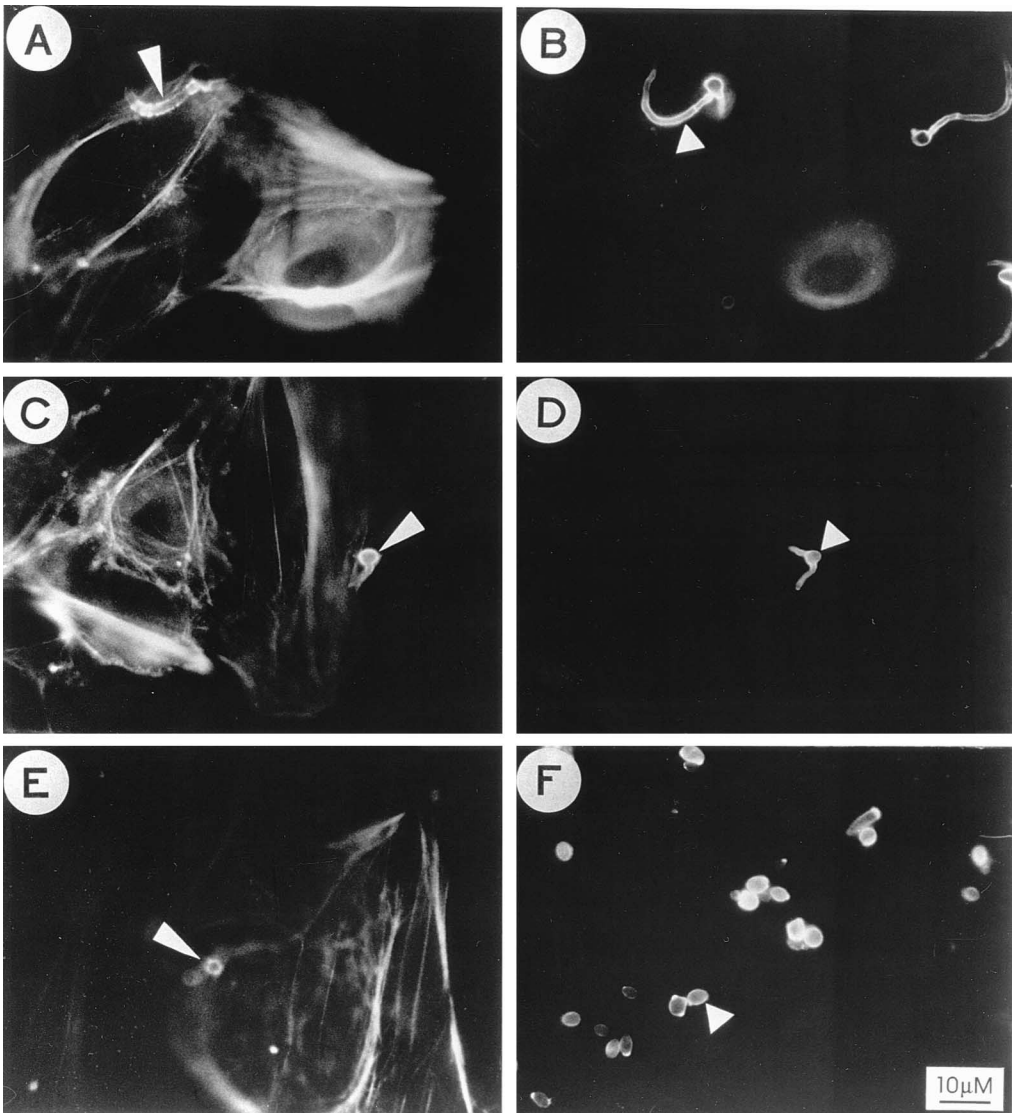

FIG. 1. *C. albicans* induces actin polymerization by endothelial cells. Endothelial cells were incubated with *C. albicans* for 2 h and then fixed and permeabilized. Endothelial cell microfilaments were visualized with BODIPY 581/591-phalloidin (A, C, and E), and the *C. albicans* cells were visualized with uvitex 2B (B, D, and F). (A and B) Live *C. albicans*. (C and D) Periodate-killed germinated organisms. (E and F) Periodate-killed blastospores. Each pair of micrographs represents the same microscopic field. The thin arrowheads indicate the condensation of actin around the organisms, and the broad arrowheads indicate the internalized organisms.

cell phagocytosis of both live and periodate-killed organisms (Fig. 2). After being ingested, some organisms were observed to be in phagocytic vacuoles that opened to the ablumenal sides of the endothelial cells (Fig. 2B). Thus, it appeared that the organisms had passed through the endothelial cells. This process did not require metabolically active *C. albicans* because this phenomenon was observed even with dead organisms. An alternative explanation for this phenomenon is that the endothelial cells had migrated over *C. albicans* so that the organisms were ingested by the ablumenal surface of the endothelial cells.

To determine if non-*albicans* species of *Candida* were also phagocytized, the endothelial cells were incubated with *C. tropicalis* and *T. glabrata* and the endothelial cell microfilaments were visualized. We saw no evidence of actin polymerization around either of these organisms, even when up to 10^6 organisms were added to each coverslip of endothelial cells. Therefore, neither organism was phagocytized by endothelial cells.

Exposing the endothelial cells to cytochalasin D almost completely disrupted the array of microfilaments and prevented the formation of the actin tubes usually seen in normal endothelial cells (Fig. 3A and B). Examination of these cells by transmission electron microscopy confirmed that disruption of the microfilaments with cytochalasin D blocked endothelial cell phagocytosis of the organisms (data not shown).

Phalloidin inhibits the depolymerization of actin and interferes with some microfilament-dependent processes in endothelial cells, such as the shape changes involved in endothelial cell retraction (1, 17, 30). Thus, we examined the effect of this compound on the phagocytosis of *C. albicans* by endothelial cells. Exposing the endothelial cells to 1 μM phalloidin did not prevent the formation of actin tubes around the organisms (data not shown). Therefore, inhibition of actin depolymeriza-

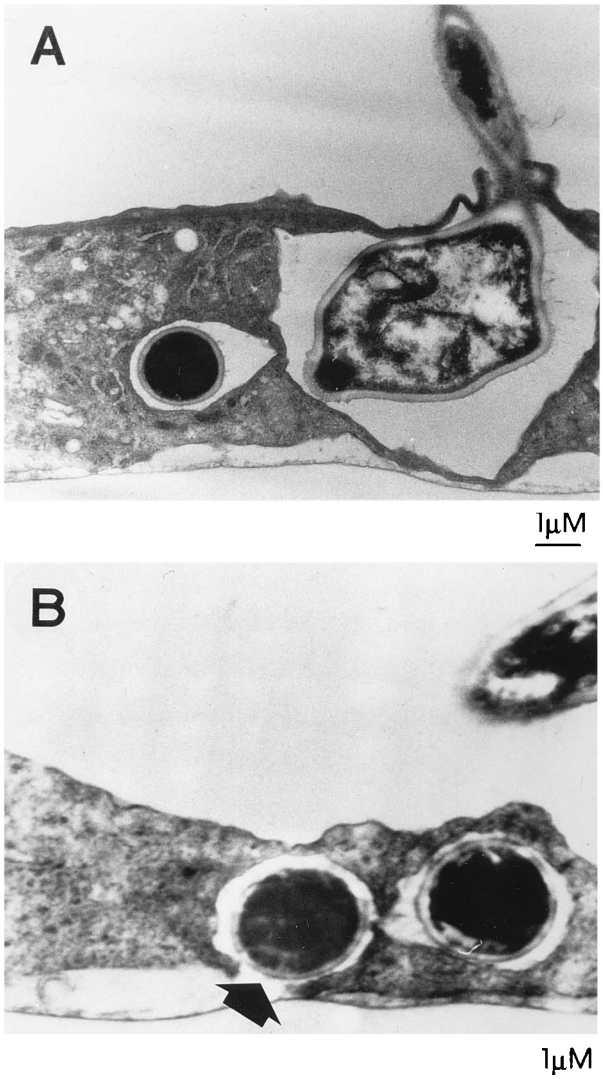

FIG. 2. Internalization and transcytosis of killed *C. albicans* by endothelial cells. Endothelial cells were incubated with periodate-killed germ tubes (A) or blastospores (B) for 2 h and processed for transmission electron microscopy. The arrow in panel B indicates a phagocytic vesicle containing a blastospore that has opened to the ablumenal surface of the endothelial cell.

tion with phalloidin did not alter endothelial cell phagocytosis of *C. albicans.*

Disruption of endothelial cell microtubules impairs phagocytosis of *C. albicans.* Phagocytosis of *C. albicans* caused no visible changes in the architecture of the endothelial cell microtubules (Fig. 4A and B). When the endothelial cells were incubated in 2 μM nocodazole, the endothelial cell microtubules almost completely depolymerized (data not shown). This depolymerization of the microtubules inhibited phagocytosis of the organism. After incubation with nocodazole, almost no organisms were phagocytized by the endothelial cells as determined by transmission electron microscopy (data not shown). Similarly, when nocodazole-treated endothelial cells were stained for microfilaments, the usual polymerization of actin around *C. albicans* was not seen, since the organisms were not ingested (Fig. 4C and D). Exposure to nocodazole also caused the actin stress fibers to become thicker and more prominent, while the microfilaments in the peripheral band disappeared in most endothelial cells (Fig. 4C).

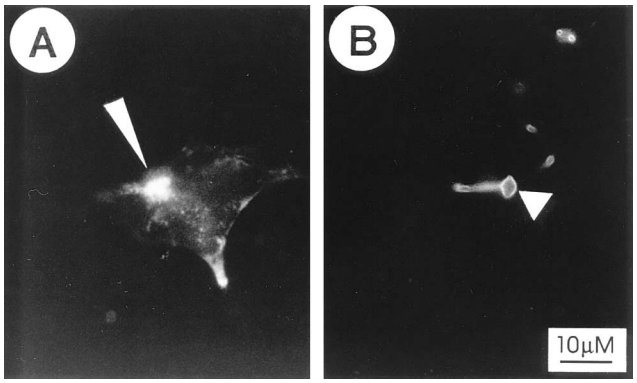

FIG. 3. Cytochalasin D disrupts actin polymerization around *C. albicans* by endothelial cells. Endothelial cells were incubated in 0.4 μM cytochalasin D for 20 min, after which live blastospores of *C. albicans* in the same concentration of cytochalasin D were added for 2 h. (A) Endothelial cell microfilaments, stained with BODIPY-phalloidin. (B) The same field as that in panel A, showing *C. albicans* cells visualized with uvitex. The arrowheads indicate the organism attached to the endothelial cell.

The endothelial cells were also incubated with 1 μM taxol to determine if enhancing the polymerization of microtubules would alter the phagocytosis of *C. albicans*. This agent did not alter the pattern of actin polymerization around the organism or affect endothelial cell phagocytosis (data not shown).

Phagocytosis of *C. albicans* is a major determinant of endothelial cell injury. To ascertain if direct contact of *C. albicans* cells with endothelial cells was required for injury to the endothelial cells, the organisms were separated from the endothelial cells by cell culture inserts. The *C. albicans* cells germinated normally when grown in this manner but caused no detectable endothelial cell injury. The specific release of ^{51}Cr from endothelial cells exposed to the organisms on filter inserts was $-3.0\% \pm 2.1\%$, compared with $33.2\% \pm 0.4\%$ when the endothelial cells were exposed to *C. albicans* directly ($P < 0.001$).

Next, the relationship between endothelial cell phagocytosis of *C. albicans* and endothelial cell injury was examined by using the inhibitors of cytoskeletal function. As can be seen from Fig. 5A, cytochalasin D decreased the amount of *Candida*-induced endothelial cell injury in a dose-dependent manner; exposure to 0.4 μM cytochalasin D led to a 71% reduction in the specific release of ^{51}Cr. Nocodazole, which also blocked the phagocytosis of *C. albicans*, significantly reduced endothelial cell injury caused by the organism (Fig. 5B). Similarly, another microtubule-disrupting agent, colchicine, protected endothelial cells from *Candida*-induced injury but to a lesser extent (Table 1). In contrast, phalloidin and taxol, which did not inhibit the phagocytosis of *C. albicans*, had no effect on endothelial cell injury (Table 1).

In additional experiments, we determined that the protective effects of cytochalasin D, nocodazole, and colchicine were due to their action on the endothelial cells and not on the organisms. Because candidal germination requires the coordinated activities of multiple cellular processes within the organism (28), it was used as an indicator of the integrated metabolic activity of the *C. albicans* cells. Live blastospores of *C. albicans* were incubated for 3 h with endothelial cells in the presence of the maximal concentration of these inhibitors used in the experiments, after which the lengths of the germ tubes were measured. As can be seen from Table 2, neither cytochalasin D nor colchicine had a significant effect on germ tube length.

Endothelial cell injury requires live organisms. Because pe-

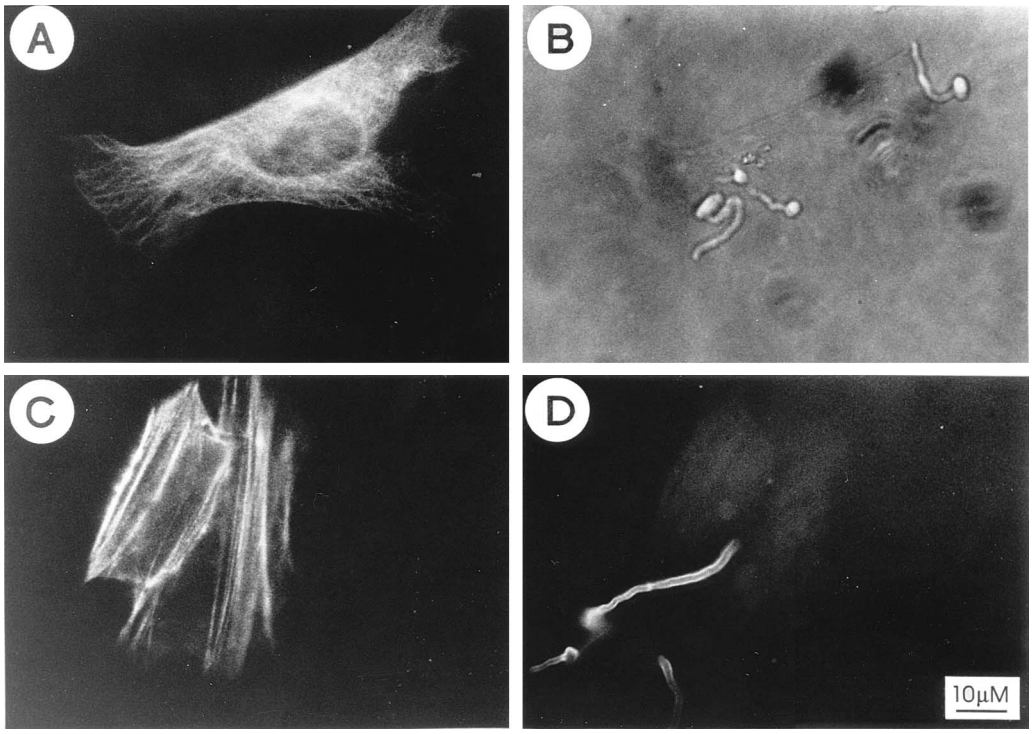

FIG. 4. Microtubules are involved in the phagocytosis of *C. albicans* by endothelial cells. (A) Indirect immunofluorescence of the microtubules of an endothelial cell in contact with *C. albicans* for 2 h. (B) The same field as that in panel A, viewed by bright-field microscopy, showing *C. albicans*. (C and D) Endothelial cells were incubated in 2 μM nocodazole, and then live blastospores suspended in the same concentration of nocodazole were added and incubation was continued for 2 h. (C) Endothelial cell microfilaments stained with BODIPY-phalloidin. (D) *C. albicans* in the same field as that in panel C, visualized with uvitex. Endothelial cell microtubules were not altered by contact with *C. albicans*; however, disruption of the microtubules with nocodazole inhibited the condensation of endothelial cell microfilaments around the organisms.

riodate-killed organisms were phagocytized by endothelial cells, we examined whether ingestion of dead organisms would result in endothelial cell injury. After being incubated with endothelial cells for 3 h, neither periodate-killed blastospores nor germinated organisms caused detectable damage (the specific release of ^{51}Cr induced by dead blastospores and germ tubes was $-1.2\% \pm 2.0\%$ and $-2.6\% \pm 1.4\%$, respectively). Thus, endothelial cell injury was not the result of phagocytosis per se but was due to a factor associated with live *C. albicans* cells.

Previously, we have found that germination of *C. albicans* was required for the organism to injure endothelial cells. The degree of endothelial cell injury is proportional to the average germ tube length of the organism (14). For this reason, we hypothesized that allowing the organisms to germinate before adding them to the endothelial cells would increase the amount of *Candida*-induced endothelial cell injury. Therefore, we compared the endothelial cell injury caused by organisms pregerminated in RPMI with that caused by control organisms that were added to the endothelial cells as blastospores and allowed to germinate on the monolayer. Pregerminated organisms had germ tubes that were 142% longer than those of control organisms ($P < 0.001$) after a 90-min exposure to endothelial cells (Fig. 6). Even though the pregerminated organisms were more than twice as long as the control organisms, they caused only 69% more endothelial cell injury ($P < 0.001$) at this time point. By 3 h, the effect of pregermination was no longer apparent, since pregerminated organisms caused damage equivalent to that caused by organisms germinated on the endothelial cells ($P = 0.32$), even though the average germ tube length of the pregerminated organisms remained significantly longer ($P < 0.001$).

DISCUSSION

Endothelial cell phagocytosis of *C. albicans* is unlike phagocytosis of species of *Rickettsia* and *Salmonella*. In addition to phagocytizing *C. albicans*, endothelial cells have been reported to ingest *Staphylococcus aureus* (18), *Rickettsia prowazekii* (39), and the Re mutant of *Salmonella minnesota* (32) in vitro. Furthermore, the phagocytosis of group B streptococci by pulmonary endothelial cells has been observed both in vitro and in vivo (15). Interestingly, endothelial cells did not ingest *C. tropicalis* or *T. glabrata*. Similarly, we have found that neither encapsulated nor acapsular *Cryptococcus neoformans* cells are phagocytized by endothelial cells in the absence of serum (unpublished data). Thus, endothelial cell phagocytosis is a selective process, and not all organisms are able to trigger this phenomenon. This selectivity is evidence that the phagocytosis of *C. albicans* likely involves the binding of specific molecules on the surface of the organism to receptors on the endothelial cell.

The phagocytosis of *C. albicans* by endothelial cells did not require metabolically active organisms since killed *C. albicans* cells were still ingested. The phagocytosis of *S. aureus* by endothelial cells is similar in this regard (18). In contrast, *R. prowazekii* induces its own phagocytosis by a different mechanism since only metabolically active organisms are ingested by endothelial cells (39). We observed also that endothelial cell phagocytosis of *C. albicans* occurred in the absence of serum,

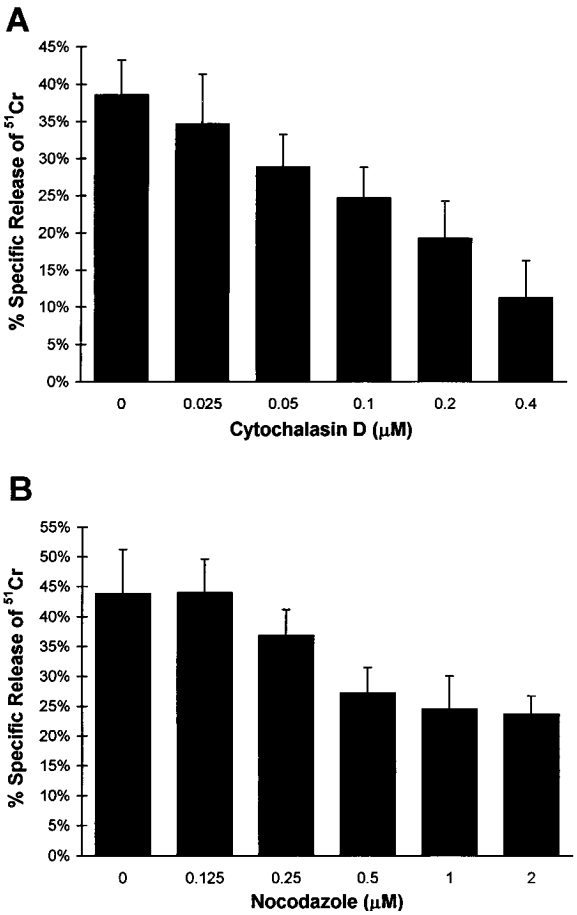

FIG. 5. Cytochalasin D and nocodazole reduce endothelial cell injury by *C. albicans*. Endothelial cells in 24-well plates were loaded with ^{51}Cr, incubated with the indicated concentrations of cytochalasin D (A) or nocodazole (B) for 20 min, and then exposed to live *C. albicans* blastospores in the same concentrations of inhibitor for 3 h. The amount of *Candida*-induced endothelial cell injury was measured as the specific release of ^{51}Cr. The results are means ± standard deviations of at least three experiments.

whereas Ryan et al. (32) found that C1q is required for the efficient phagocytosis of *S. minnesota* by endothelial cells. Thus, the phagocytosis of *C. albicans* is likely mediated by a different endothelial cell receptor(s) from the one(s) involved in the ingestion of *S. minnesota.*

We observed that actin polymerized around the germ tube portions of live organisms more frequently than it did around the blastospore portions. In addition, little actin polymerization around the organisms was seen until after the organisms had germinated. Therefore, it is likely that the candidal ligand(s) that is recognized by endothelial cell receptors either binds with higher affinity or is present in higher numbers on the germ tubes. Alternatively, entirely different ligands may be present on the surface of germinated organisms by comparison with those on blastospores. For instance, the surface antigens of *C. albicans* are known to change significantly when the organism undergoes transformation from the blastospore to the germinated form (3, 27).

TABLE 1. Effects of inhibitors of cytoskeletal function on endothelial cell injury by *C. albicans*

Agent (concn)	% Inhibition of endothelial cell injury (mean ± SD)[a]	P^a
Cytochalasin D (0.4 μM)	71.0 ± 12.1	<0.001
Phalloidin (1 μM)	−1.9 ± 17.5	>0.5
Nocodazole (2 μM)	42.1 ± 11.5	<0.001
Colchicine (1 μM)	29.3 ± 7.6	<0.001
Taxol (1 μM)	−3.7 ± 6.9	>0.5

[a] By comparison with results for control endothelial cells infected with *C. albicans* in the absence of inhibitor. The mean *Candida*-induced specific release of ^{51}Cr from control cells ranged from 38.6 to 44.3%. Each inhibitor was tested in three to five separate experiments.

TABLE 2. Effects of inhibitors of cytoskeletal function on germ tube elongation of *C. albicans*

Agent (concn)	Germ tube length (μm)[a]	P^b
HBSS	39.0 ± 15.8	
Cytochalasin D (0.4 μM)	40.8 ± 15.5	>0.5
Nocodazole (2 μM)	45.7 ± 23.1	0.05
Colchicine (2 μM)	43.2 ± 15.7	0.18

[a] Values are means ± standard deviations.
[b] By comparison with results for organisms grown on endothelial cells in HBSS.

The phagocytosis of *C. albicans* requires functional microtubules and microfilaments. In addition to blocking endothelial cell phagocytosis of *C. albicans*, cytochalasins have been shown to inhibit the internalization of *S. aureus* (18), *R. prowazekii* (39), and group B streptococci (15) by endothelial cells. However, it is likely that these different organisms stimulate disparate patterns of actin polymerization. For instance, internalized *Rickettsia conorii* has been found to induce the formation of actin tails in endothelial cells (35), whereas the pattern of actin polymerization stimulated by *C. albicans* was distinctly different, with tubes of actin condensing around these organisms.

Phalloidin and its derivative, phallacidin, inhibit microfilament depolymerization and prevent endothelial cells from changing shape in response to a variety of soluble stimuli (1, 17, 30). However, the blocking of microfilament remodelling with phalloidin did not interfere with the ingestion of *C. albicans*. Therefore, it is likely that phagocytosis of this organism occurred in the absence of significant microfilament depolymerization and that the endothelial cell actin that polymerized

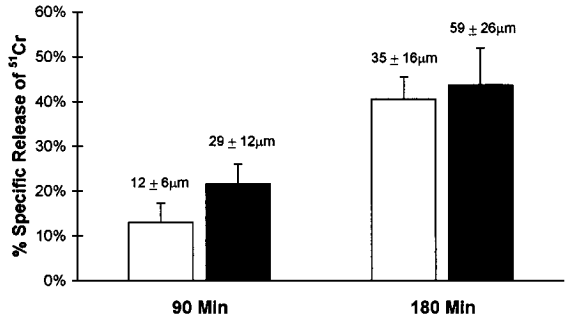

FIG. 6. Germination of *C. albicans* prior to addition of the organism to endothelial cells only slightly increased endothelial cell injury. *C. albicans* cells were added to endothelial cells as either blastospores (open bars) or germ tubes (solid bars), after which endothelial cell injury was determined at the indicated times (the results are means ± standard deviations of three experiments). The numbers above each column indicate the average length of the germ tubes ± the standard deviation at each time point.

around the organism originated from a pool of unpolymerized actin (G-actin).

Microtubules must play a pivotal role in the phagocytosis of *C. albicans*, since depolymerizing them with nocodazole prevented the formation of actin tubes around the organism and blocked phagocytosis. Similarly, Donnenberg et al. (7) found that the inhibiting of microtubule function blocked the phagocytosis of enteropathogenic *Escherichia coli* by epithelial cells. However, microtubules are not involved in the phagocytosis of all particles by endothelial cells. For example, Steffan et al. (34) reported that the phagocytosis of latex beads by hepatic endothelial cells was not inhibited by the depolymerization of microtubules.

Microtubules direct the transport of certain receptors to and from the cell surface (36). For instance, agents that depolymerize microtubules markedly reduce the number of tumor necrosis factor alpha receptors on endothelial cells (6). Therefore, one explanation for the inhibitory effect of nocodazole on the phagocytosis of *C. albicans* is that the agent may decrease the number of endothelial cell receptors that mediate the ingestion of the organism. However, the expression of these putative receptors for *C. albicans* apparently is not inhibited by microtubule stabilization with taxol, since this agent had no effect on endothelial cell phagocytosis of *C. albicans.*

Endothelial cell phagocytosis of *C. albicans* may be a mechanism by which hematogenously disseminated organisms invade the parenchyma of target organs. The microbially directed formation of microfilaments has been shown to be involved in the transcellular spread of certain bacteria as well as in the internalization of extracellular organisms in epithelial cells (4, 11, 20, 37). In this regard, it was of interest to determine that some of the *C. albicans* cells were found in phagocytic vacuoles that opened to the ablumenal sides of the endothelial cells. This phenomenon likely represented transcellular passage of the organism. It was a passive process on the part of the *C. albicans* cells since it occurred even with periodate-killed blastospores (Fig. 2B). Therefore, it is possible that target organ invasion by blood-borne *C. albicans* is facilitated by endothelial cell phagocytosis of the organism followed by fusion of the phagocytic vacuole with the ablumenal plasma membrane. Another mechanism by which *C. albicans* may egress from the intravascular compartment is to injure the endothelial cells and expose the subcellular matrix.

Mechanisms of endothelial cell injury by *C. albicans*. The results of the experiments in which *C. albicans* cells were separated from endothelial cells by filter inserts, combined with our previous findings that culture filtrates from *C. albicans* do not cause endothelial cell damage (10), strongly suggest that the activities of any candidal products released into the medium are not sufficient to cause endothelial cell injury by themselves. Although *C. albicans* cells secrete phospholipases and proteases that can potentially damage endothelial cells, it is unlikely that these enzymes have significant activity in the buffered medium, since their pH optima are less than 4.5 (2, 33). This requirement for an acidic pH may explain why endothelial cell injury was markedly reduced when internalization of the organisms was inhibited. When all or part of the organism is contained in a phagocytic vacuole, the pH within the vacuole is likely to be low enough for the candidal enzymes to be active. Furthermore, any candidal proteases and phospholipases secreted into the vacuole would be able to accumulate to concentrations toxic to the endothelial cell. Although it is known that the pH inside the phagocytic vacuoles of epithelial cells is <7 (13), to our knowledge, the pH of the phagocytic vacuoles of endothelial cells has not yet been determined.

The finding that only live organisms were able to cause endothelial cell injury provides further evidence that endothelial cell injury is triggered by the secretion of cytotoxic products by *C. albicans*. It is likely that these cytotoxic substances are secreted mainly by germinating organisms for two reasons. First, substances that inhibit germination, such as sublethal concentrations of antifungal agents, also block *Candida*-induced endothelial cell injury (14). Second, the time course of candidal germination and germ tube elongation on endothelial cells parallels the time course of endothelial cell injury (10).

In the current study, we found that organisms that were pregerminated prior to being added to the endothelial cells caused less endothelial cell injury than would be expected on the basis of the lengths of their germ tubes. Therefore, once germination has occurred, the amount of *Candida*-induced endothelial cell injury is dependent mainly on the duration of exposure to the organisms rather than on the actual lengths of the germ tubes. Possible explanations for this finding are that endothelial cell phagocytosis of *C. albicans* is the rate-limiting step in the development of endothelial cell injury and/or that exposure to endothelial cells is required to induce *C. albicans* germ tubes to express the factor(s) responsible for the endothelial cell damage.

The phagocytosis of *C. albicans* and the subsequent development of endothelial cell injury are possible mechanisms by which this organism may escape from the circulation and invade the tissue parenchyma. This process is likely mediated by the binding of the organism to endothelial cell receptors which are transported to the endothelial cell surface by microtubules. These receptors are specific for *C. albicans*, since non-*albicans* species of *Candida* do not trigger the phagocytic process. Activation of these receptors causes endothelial cell microfilaments to polymerize around the organism and to draw it into the endothelial cell. An ingested organism can then escape from the endothelial cell by fusing the phagocytic vacuole with the ablumenal plasma membrane or by killing the endothelial cell. The regulation of these processes may be a method to prevent hematogenously disseminated candidal infections.

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