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Migration of the Fungal Pathogen *Candida albicans* across Endothelial Monolayers

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Migration of the fungal pathogen *Candida albicans* across the endothelial cell layer is considered a prerequisite for the invasion of multiple organs occurring in systemic candidiasis. We developed an experimental system in which *C. albicans* migrates from a luminal compartment across a monolayer of bovine aortic endothelial cells on a porous filter support to an abluminal compartment. In this system, a *C. albicans* wild-type strain (ATCC 10261) traverses the endothelial monolayer in a time-, glucose-, and cell concentration-dependent manner. A mutant derivative unable to grow and form hyphae (SGY-243) migrates at a reduced rate. Concomitant to transendothelial migration, the permeability of the endothelial monolayer for dextran diffusion markers is significantly increased. This increase in transendothelial exchange occurs before fungal cells are detectable in the abluminal compartment and is time, glucose, and cell concentration dependent. A mutant strain (hOG301) unable to interact with endothelial cells does not alter endothelial permeability. Thus, transendothelial migration of *C. albicans* is able to damage the barrier function of an endothelial monolayer. Our experimental system, which reflects key stages of transendothelial migration of *C. albicans* including adherence and passage across endothelial cells and the extracellular matrix, may be a useful model for comparisons of transendothelial migration characteristics of *Candida* strains.

A range of conditions including immunodeficiency and diabetes predisposes patients to infections by the fungal pathogen *Candida albicans* (24). In compromised patients, *C. albicans* is able to disseminate hematogenously and to cause life-threatening systemic mycoses by colonization of various organs. Passage across the vascular cell lining is a prerequisite for organ colonization and consists of several processes, including adherence to endothelial cells, migration across the endothelium, and penetration of the extracellular matrix.

Adherence to endothelial cells is mediated in part by a *C. albicans* glycoprotein known to bind the complement fragment iC3b (10, 11, 22). Additional binding may be due to a glycoprotein able to bind fibronectin, whose receptor activity is blocked by peptides containing an Arg-Gly-Asp sequence (21, 23, 25). Following adherence, fungal cells can be phagocytosed by endothelial cells, as has been visualized by in vitro systems; functional microfilaments and microtubules of the host cell are required for this process (8, 26). The exit of ingested fungal cells on the basolateral side is thought to occur by exocytosis (8). Although phagocytosis and exocytosis of the yeast form of *C. albicans* have been observed, it is not clear if the hyphal growth forms seen in electron micrographs of adhering fungi (26) play a relevant role in transmigration. Damage to or contraction of the endothelial layer may allow direct access of *Candida* cells to the extracellular matrix on the basolateral side of the endothelium (20). Finally, traversal of the extracellular matrix may require fungal proteins binding to fibronectin and other matrix components (4), as well as hydrolytic enzymes including phospholipases and proteases (14, 15).

It appears likely that an efficient pathogen such as *C. albicans* will have optimized each of the foregoing processes to achieve maximal overall rates of migration across the endothelial cell layer. Because we considered that the characteristics of each individual process would not necessarily reflect overall migration rates, we established and characterized an in vitro system in which the passage of *C. albicans* across an endothelial monolayer can be monitored in toto. We demonstrate here that during transendothelial migration of *C. albicans* the barrier function of the endothelial monolayer is damaged significantly.

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MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** The *C. albicans* strains used were ATCC 10261 (prototroph) and mutants SGY-243 (*ade2*/*ade2 ura3*::*ADE2*/*ura3*::*ADE2*) (17) and hOG301 (13). Strain SGY-243, which is phenotypically Ura$^-$, was grown in SD minimal medium (29) supplemented with 10 mg of uridine per ml. hOG301 grows constitutively in filamentous form (predominantly as pseudohyphae). Strains were grown routinely in liquid YPD medium (29) at 30°C, and viable-cell counts were determined by plating dilutions on YPD plates and expressed as CFU. Crude cell wall-membrane fractions of strains ATCC 10261 and hOG301 were prepared by first growing cells to an optical density at 600 nm of 1.5 in YPD medium and then washing and resuspending them in buffer (100 mM Tris HCl [pH 7.2], 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100); cells were broken by agitation (5 min) in a homogenizer (Braun, Melsungen, Germany) in the presence of an equal volume of glass beads (0.45-mm diameter). A crude cell wall-membrane fraction was obtained by centrifugation at 2,000 × *g* for 10 min, washed with water, and resuspended in buffer.

Isolation of BAEC. We isolated bovine aortic endothelial cells (BAEC) from aortas of freshly slaughtered calves according to the method of Booyse et al. (2), as described previously (32). Briefly, aortas were cut lengthwise and washed with phosphate-buffered saline (140 mM NaCl, 4 mM KCl, 1 mM $Na_2HPO_4 \cdot 2H_2O$, 1 mM KH_2PO_4, and 12 mM glucose; pH 7.4). The adventitia was removed, and the remaining parts of intima and media were clamped into the aluminum frame of a small tub. The intima with the endothelium formed a well, which was filled with 5 ml of dispase (0.5 mg/ml) (Boehringer Mannheim). After 15 min of incubation at 37°C, the enzyme solution was removed, and the endothelial cells were collected by washing the intima with 6 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma D5546) containing fetal calf serum (FCS) at a final concentration of 20% (Flow Laboratories, Meckenheim, Germany). The cells were plated in three 35-mm-diameter wells which had been pretreated with 0.2% gelatin for 30 min at room temperature. The next day, the cells were washed with DMEM and cultured with 10% FCS–DMEM at 37°C in an atmosphere of 5% CO_2 and 95% air. For subculturing, the cells were released with trypsin (0.5 mg/ml), harvested by centrifugation (100 × *g*, 10 min), and then plated at a dilution of 1:7.

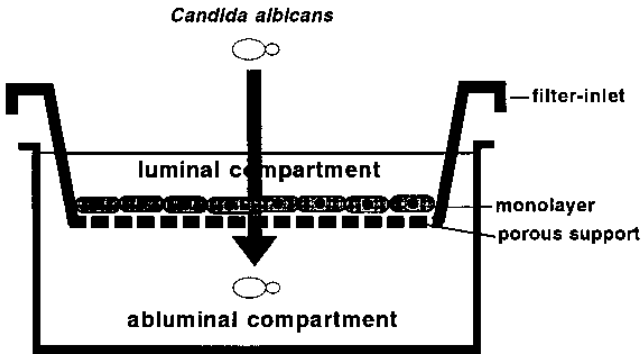

FIG. 1. Schematic representation of the assay for transendothelial migration of *C. albicans*. A confluent monolayer of BAEC on a porous filter separates a luminal from an abluminal compartment. A defined number of *C. albicans* cells is used to inoculate the luminal compartment, and the appearance of viable fungal cells on the abluminal side is determined.

The cells were identified as endothelial cells by their cobblestone morphology, by immunostaining using anti-human factor VIII (Red Cross, Amsterdam, The Netherlands) (16), and by the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate-labelled acetylated low-density lipoprotein (Paesel, Frankfurt, Germany) (31).

Migration assay. *C. albicans* migration through a BAEC monolayer was determined by using six-well plates with polycarbonate filter inserts (3.0-μm pores; 4.71-cm^2 area) (Costar Europe, Badhoevedorp, The Netherlands). In the standard assay, endothelial cells were plated on the filters at 80,000 cells per cm^2 in DMEM (no FCS) containing 5.5 mM (0.1%) glucose. DMEM with different glucose concentrations was prepared by adding glucose to 22 mM or by preparing DMEM without glucose. Following incubation at 37°C for 2 to 3 days, confluency was reached at a cell density of 100,000 to 120,000/cm^2. In parallel experiments, collagen filter inserts (Transwell-Col; Costar Europe) were used, which allowed the monitoring of confluency by light microscopy. The luminal compartment (1.5 ml) of the confluent endothelial monolayer was inoculated with defined numbers of *C. albicans* cells (10^6 CFU/ml in the standard assay) and allowed to stand at 37°C in a cell culture incubator in an atmosphere of 0.5% CO_2 for different times. As judged by the color of the pH indicator in DMEM, the pH of the medium was not affected during any of the migration assays. At the end of each incubation, the abluminal medium (2.5 ml) was collected and the compartment was washed twice with 2 ml of DMEM. *C. albicans* viable-cell counts in the abluminal compartment were determined by plating dilutions on YPD plates.

Transendothelial exchange (TEE). Endothelial permeability was determined as described previously (32). In brief, endothelial cells were seeded on polycarbonate membranes (3.0-μm pores; 4.71-cm^2 area) (Costar Europe). At 0 min, 10 μM fluorescein isothiocyanate-labelled dextran with an M_r of 40,000 (FD40) (Sigma) was added to the luminal compartment. The paracellular diffusion of FD40 across the endothelial monolayer from the luminal to the abluminal side was taken as a parameter for endothelial integrity. Samples were measured in a 650-40 fluorimeter (Perkin-Elmer, Überlingen, Germany), using 495-nm excitation and 530-nm emission wavelengths.

Electron microscopy. Endothelial monolayers on polycarbonate filter supports that had been incubated with *C. albicans* were washed with Hanks balanced salt solution and fixed with 2.5% glutaraldehyde in 0.19% cacodylate buffer (pH 7.3). Samples were treated with 2% OsO_4–2% uranyl acetate and finally embedded in Epon. Slices (0.1 μm thick) were prepared and examined in a Siemens EM1A electron microscope; slices showing the filter support were used to define the luminal and abluminal (filter-adhering) sides of endothelial cells.

Statistics. Data were calculated from the results of three different experiments for each of n cell preparations. Each cell preparation represents the cells from one individual (calf) and is taken as $n = 1$. Data are given as means ± standard deviations of the means of n different cell preparations. P values were calculated by using the t test (InStat; GraphPad Software Inc.), and values of ≤ 0.05 were considered significant.

RESULTS

Assay for transendothelial migration of *C. albicans*. We used an experimental system in which a confluent monolayer of bovine endothelial cells on a porous filter support separates a luminal and an abluminal compartment containing DMEM without FCS (Fig. 1). The luminal compartment was inoculated with a defined number of *C. albicans* cells, and after standing at 37°C in a cell culture incubator, the appearance of fungal cells in the abluminal compartment was monitored by the determination of viable-cell counts. In pretests, we had determined that a pore size of 3.0 μm for the filter support allows the passage of *C. albicans* wild-type strain ATCC 10261 without permitting significant leakage of endothelial cells.

The conditions of the standard migration assay caused *C. albicans* ATCC 10261 to grow slowly, leading to only a fourfold increase in culture density during 25 h (Fig. 2A). Viable-cell counts did not increase during this time but slightly decreased initially (Fig. 2B), since individual yeast cells developed into short pseudohyphae consisting of four to six elongated yeast cells that remained attached and aggregated occasionally. Similar growth characteristics were observed in the presence or absence of an endothelial monolayer. Variation of the glucose content of the DMEM from the standard concentration of 5.5 mM (0.1%) to 0 or 22 mM (0.4%) did not have a significant impact on fungal growth (Fig. 2C and D). However, the absence of glucose allowed the slow formation of true hyphae: about 30% of yeast cells had formed germ tubes after 5 h, and 50% of cells were in the form of true hyphae consisting of four to six hyphal compartments after 25 h. In contrast, cells grown with 5.5 or 22 mM glucose grew mainly as short pseudohyphae. We did not determine if the relatively slow growth (about two doublings in cell mass) and morphogenesis characteristics of *C. albicans* during the migration assay were caused, e.g., by the lack of aeration or components of the DMEM.

Transendothelial migration characteristics. Virtually all cells of the wild-type strain ATCC 10261 appeared to adhere tightly to endothelial cells already after 1 h of incubation, since they could not be detached by a mild perturbation of the medium, and typical stages of phagocytosis were observed for most cells by electron microscopy (see below).

The presence of an endothelial monolayer clearly retarded but did not abolish the appearance of *C. albicans* in the abluminal compartment (Fig. 3). The requirement of migration across the monolayer reduced concentrations of viable fungal cells in the abluminal compartment at any time of the assay (Fig. 3 and Table 1). An increase in the inoculum size from 0.5×10^6 to 2.0×10^6 cells per ml led to a proportional increase in fungal migration; at a *C. albicans* concentration of 1.0×10^6 cells per ml, the initial ratio of fungal to endothelial cells in the system was approximately 3. The addition of glucose significantly stimulated the transendothelial migration of *C. albicans* (Table 2). This effect cannot be due to stimulation of fungal growth, which is identical at 0 and 5.5 mM glucose (Fig. 2D); only at 22 mM glucose could growth have increased cell counts on the basolateral side. An acidification of the medium able to damage the monolayer was not observed.

Electron microscopy. Electron microscopy was used to clarify the pathway by which *C. albicans* traversed the endothelial monolayer. Typical stages of migration are shown in Fig. 4. At an initial stage of *Candida*-endothelial cell interaction, adhering yeast cells were shown to elicit the formation of pseudopodia (Fig. 4A), which elongated further and at a later stage engulfed fungal cells (Fig. 4B). Following fusion of the endothelial pseudopodia, yeast cells were found in the interior of cells, surrounded by the membrane of the phagocytic vacuole (Fig. 4C). As expected, staining with ruthenium red stained only the plasma membrane and not the membrane of the presumed vacuolar membrane, excluding the possibility that the image of Fig. 4C was due to a yeast cell at the cell surface. Images of the exit of fungal cells on the abluminal side were rarely obtained. However, in all cases, evading cells were shown to form hyphal extensions (Fig. 4D), suggesting that hyphal growth is the major route for the exit of *C. albicans* from infected endothelial cells. In contrast, the initial phases of

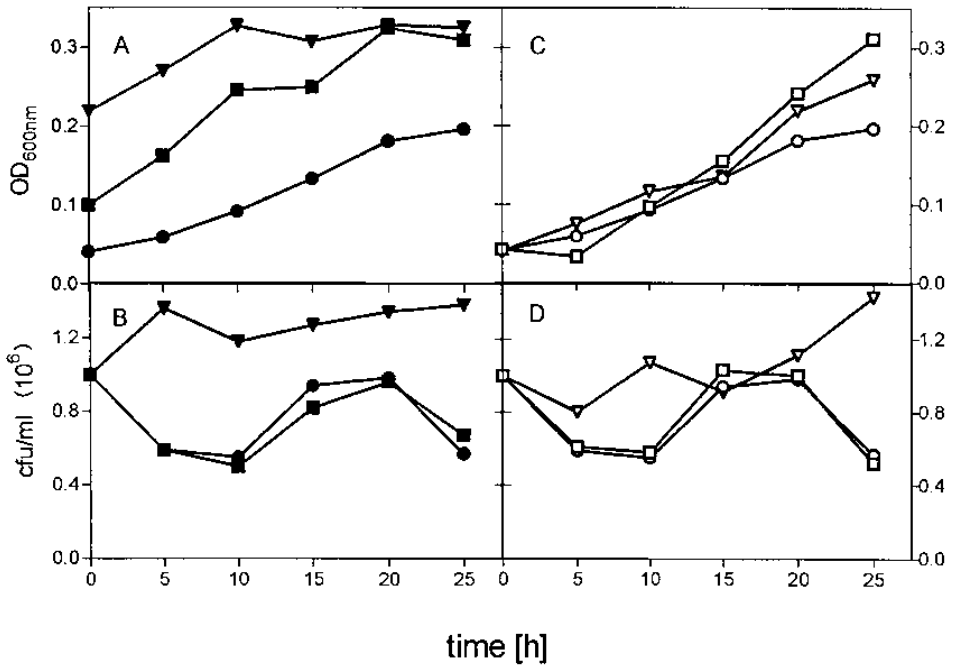

FIG. 2. Growth of *C. albicans* in cell culture media. Cell culture wells containing DMEM with and without glucose were inoculated with 10^6 CFU of *C. albicans* strains per ml and incubated at 37°C without agitation. At different times, the culture density and viable fungal cells were determined. Values are the means of two independent measurements, which differed by <20%. Growth of *C. albicans* ATCC 10261 (●), SGY-243 (▼), and hOG301 (■) in DMEM (5.5 mM glucose) was compared (A and B), and growth of strain ATCC 10261 in the presence of 0 (□), 5.5 (○), and 22 (∇) mM glucose was determined (C and D). OD_{600nm}, optical density at 600 nm.

phagocytosis were observed to be associated with only the yeast growth form of *C. albicans*. Fungal cells were rarely seen traversing the interstitial space between endothelial cells during migration. Thus, the microscopic results suggest that the mechanism of transendothelial migration involves internalization of yeast cells by phagocytosis on the luminal side and exit by hypha formation on the abluminal side of endothelial cells.

Damage of endothelial cells. Endothelial integrity can be shown by a defined permeability of diffusion markers, such as fluorescein isothiocyanate-labeled dextran (5). To examine the effects of transendothelial migration of *C. albicans* on the endothelial monolayer, we investigated diffusion of FD40, a labeled dextran with a molecular mass of 40,000 Da. In these experiments, the luminal compartment was first inoculated with 10^6 *C. albicans* cells per ml (or not inoculated for controls); subsequently, the monolayer system was incubated for 6 h. Following incubation, 10 μM FD40 was added to the luminal compartment, and the appearance of FD40 in the abluminal compartment was monitored. Figure 5 shows the time dependence of TEE of FD40, calculated as the percentage of the total amount of FD40 appearing in the abluminal compartment. An increased permeability of the *C. albicans*-treated monolayer relative to that of untreated control monolayers already became apparent 120 min after pretreatment with the wild-type strain ATCC 10261 (17.0% ± 0.43% versus 8.95% ± 0.48% of controls [means ± standard deviations]).

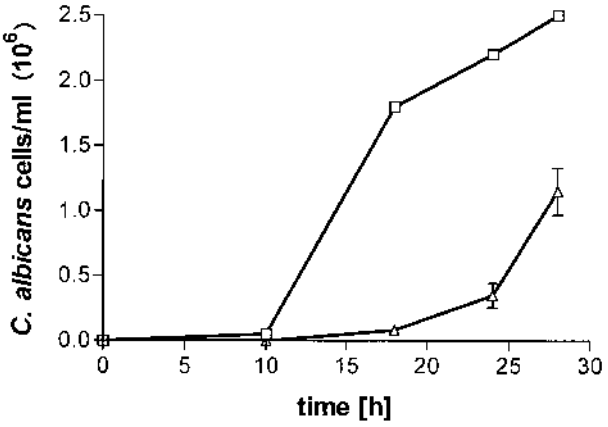

FIG. 3. BAEC monolayer as a migration barrier for *C. albicans*. *C. albicans* cells (10^5/ml) were added to the luminal compartment of a two-compartment system containing a BAEC monolayer (Fig. 1) (Δ) or not containing a monolayer as a control (□). After different times of incubation, cell concentrations of viable fungal cells in the abluminal compartment were determined.

TABLE 1. Migration of *C. albicans* across an endothelial monolayer

Strain	Viable *C. albicans* cells (mean CFU/ml ± SD)[a]	
	Luminal compartment	Abluminal compartment
ATCC 10261	0.5×10^6	21,000 ± 1,050
	1.0×10^6	37,430 ± 1,800
	2.0×10^6	85,570 ± 10,410
	1.0×10^6 (no monolayer)	461,770 ± 36,320
hOG301	1.0×10^6	0
	1.0×10^6 (no monolayer)	0
SGY-243	1.0×10^6 (no uridine)	8,000 ± 2,000
	1.0×10^6 (with uridine)	40,000 ± 5,000

[a] The luminal compartment was inoculated with *C. albicans* strains, and the appearance of viable cells on the abluminal side of the endothelial monolayer was determined after 24 h. Values are the means of 10 separate experiments using three different endothelial cell strains.

TABLE 2. Effect of glucose on transendothelial migration of *C. albicans*[a]

Inoculum for luminal compartment	*C. albicans* cells (mean CFU/ml ± SD) in albuminal compartment at the indicated glucose concern (mM)		
	0	5.5	22
0.5×10^6	ND	21,000 ± 1,050	ND
1.0×10^6	20,000 ± 2,100	37,430 ± 1,800	54,920 ± 4,800
2.0 ± 10^6	ND	85,570 ± 10,410	ND

[a] The luminal compartment was inoculated with 1.0×10^6 cells of *C. albicans* ATCC 10261 per ml, and viable cells in the abluminal compartment of the endothelial monolayer were determined after 24 h of incubation. Without an endothelial monolayer, 461,770 ± 36,320 fungal cells appeared in the abluminal compartment. Values are the means of at least 10 separate experiments using three different endothelial cell isolates. ND, not determined.

Increased *C. albicans* cell numbers from 0.5×10^6 to 1.0×10^6/ml led to greater TEE values; this was already apparent after 3 h of the assay and was still observed at 8 h (Fig. 6). However, for unknown reasons, a further increase in *C. albicans* cell numbers to 2.0×10^6 did not increase but reduced TEE values. Thus, these experiments suggest that *C. albicans* wild-type cells are able to significantly damage the endothelial monolayer already at a time when no significant numbers of fungal cells are detectable in the abluminal compartment (Fig. 3). However, fungi exiting endothelial cells are observed at this time by electron microscopy (Fig. 4D). In contrast, the hyphal mutant hOG301 did not increase the permeability of the endothelial monolayer (Fig. 5; Table 1).

As shown above, glucose is able to stimulate the transendothelial migration of *C. albicans* (Table 2). To examine an effect on TEE, the luminal compartment of the monolayer system was inoculated with 10^6 cells of *C. albicans* ATCC 10261 per ml and incubated for 6 h in the presence of increasing concentrations of glucose (0, 5.5, and 22 mM). Following this pretreatment, TEE values were determined as described above. The results demonstrate a significant increase in monolayer permeability in the presence of 5.5 mM glucose compared with that of glucose-free controls (Fig. 7). However, an increase of the glucose concentration to 22 mM did not further enhance the permeability of the endothelium.

Crude cell wall-membrane extracts of the *C. albicans* wild-type strain ATCC 10261 and of mutant hOG301 were prepared, and amounts of crude cell walls or membranes corresponding to 10^6 viable cells per ml were added to the luminal compartment. This treatment did not enhance TEE compared with that of untreated samples, suggesting that mere contact with components in *C. albicans* cell surfaces is not sufficient to damage endothelial cells and to enhance the permeability of the monolayer.

Migration characteristics of *C. albicans* mutants. Mutant hOG301 was derived from wild-type strain ATCC 10261 by chemical mutagenesis (12); it grows predominantly in filamentous form as pseudohyphae and to a lesser extent as true hyphae or as yeast cells (blastospores), at a rate similar to that of ATCC 10261 (Fig. 2). Unlike the wild-type strain, which appeared to adhere tightly to endothelial cells, hOG301 formed aggregates in the luminal compartment that appeared to be soluble and not to adhere to the endothelial monolayer. Since hOG301 did not reach the basolateral compartment after 24 h, even in the absence of endothelium (Table 1), it appears that the mutation(s) of strain hOG301 significantly alters its surface properties to promote autoaggregation, to preclude interaction with endothelial cells, and to reduce the ability to pass through the pores in the filter support. The failure of strain hOG301 to interact with endothelial cells was paralleled by the absence of an effect on the permeability of the endothelial monolayer (Fig. 5). This result supports the notion that tight adherence and phagocytosis of *C. albicans* by endothelial cells are required to damage the permeability properties of the endothelial monolayer. Lack of endothelial damage may be the cause for the reduced virulence of mutant hOG301 in animal tests (27).

Mutant SGY-243 was constructed by site-specific mutagenesis to delete the *URA3* gene; it is known that in the absence of uridine this strain is unable to grow or to develop hyphae or pseudohyphae (17). As expected, SGY-243 showed only residual growth in DMEM (Fig. 2), and microscopic inspection revealed that only yeast cells were pesent; the lack of filamentous growth prevented a decrease in CFU due to aggregation, as was observed for strains ATCC 10261 and hOG301. A cell size larger than that of strain ATCC 10261, possibly caused by a change in ploidy (9), may be the reason for the increased initial density (Fig. 2A). Using strain SGY-243, it was possible to test if growth and hyphal morphogenesis are required for migration across the endothelial monolayer. The results in Table 1 demonstrate that SGY-243 is able to migrate across the endothelial monolayer, even in the absence of uridine. In this condition, viable-cell counts on the abluminal side were reduced to 20% compared with that of a uridine-supplemented control that was able to grow and to undergo morphogenesis like the wild-type ATCC 10261. This result cannot be explained simply by fungal growth in the presence of uridine (luminal or abluminal), since viable-cell counts do not change during the assay (Fig. 2). Possibly, fungal growth and/or morphogenesis during the transit of endothelial cells is able to stimulate transendothelial migration.

DISCUSSION

A model for transendothelial migration of *C. albicans*. Migration of *C. albicans* across endothelium is considered the first step in invasion of tissue parenchyma and the establishment of systemic candidiasis. Transendothelial migration is a complex process consisting of individual steps reproduced to a large extent in the experimental model described here. Adhesion of *C. albicans* to endothelial cells has been well studied and requires fungal mannoprotein receptors for the complement component iC3b (integrin analog) and fibronectin (6, 12, 25). In our experimental system, most wild-type *C. albicans* cells had already tightly adhered after 1 h of incubation, in agreement with earlier results (22). Growth of *C. albicans* in the presence of glucose compared with glutamate was shown to significantly augment adhesion and was correlated with increased synthesis of the integrin analog (10, 11). In agreement, *C. albicans* cell counts for the abluminal compartment increased with increasing glucose concentrations in the migration assay. Since we have shown that glucose does not significantly affect growth of *C. albicans* in the culture fluid, the glucose effect could be due to increased adhesion and/or to an effect on later stages of transendothelial migration, e.g., phagocytosis. These processes, in combination with a weakened immune response and reduced circulation of peripheral blood, may be related to the enhanced disposition for candidiasis in diabetics (27). Since the absence of glucose in the medium caused *C. albicans* to form true hyphae, while pseudohyphae arose in glucose-containing media, formation of true hyphae as opposed to pseudohyphae preceding endothelial penetration appears not to facilitate transendothelial migration. Phagocytosis is the presumed route of entry into endothelial cells, which requires functional microtubules and microfilaments of

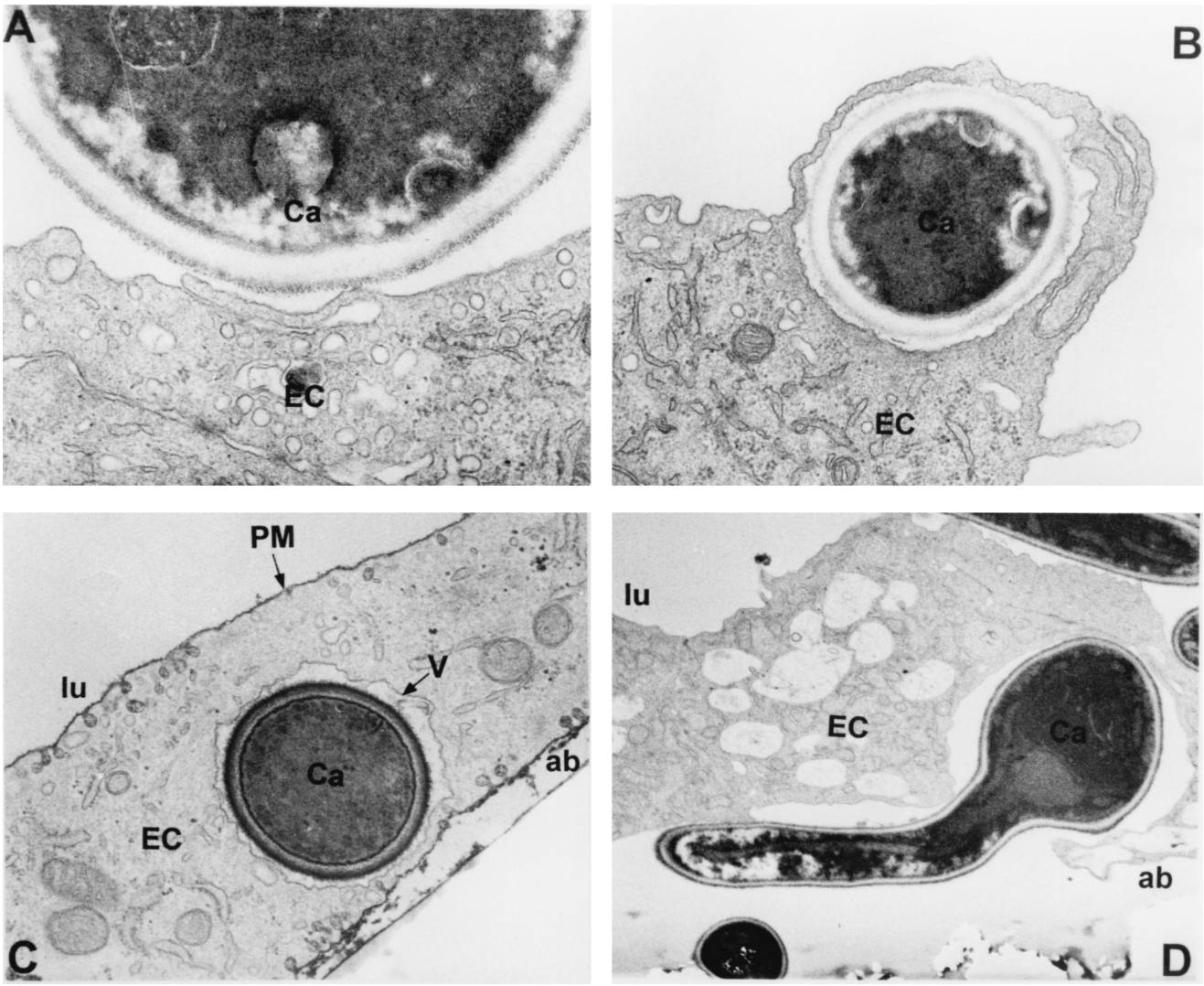

FIG. 4. Electron microscopy of *C. albicans* ATCC 10261 (Ca) interacting with endothelial cells (EC) growing as a monolayer on a filter support (Fig. 1). Interaction was allowed to proceed for 2 (A), 4 (B and C), and 6 (D) h. Photographs represent typical stages of transendothelial migration. (A) Adherence and formation of an endothelial protrusion; (B) protrusions engulfing a *C. albicans* cell; (C) internalized fungal cell; (D) *C. albicans* leaving the cell on the abluminal side. The luminal (lu) and abluminal (ab) sides of the endothelial cell, the plasma membrane on the luminal side (PM), and the vacuolar membrane (V) are indicated. Magnifications were ×48,000 (A), ×24,000 (B), ×28,500 (C), and ×10,000 (D).

the host cells and occurs in the absence of serum (8, 19, 26). As has been reported earlier (26), we observed pseudopodia arising on endothelial cells at the site of contact with *C. albicans*; pseudopodia elongated and finally engulfed fungal cells in a phagocytotic vacuole. Although during the assay pseudohyphae were slowly formed, by electron microscopy we detected almost exclusively the yeast growth form of *C. albicans* being phagocytosed by endothelial cells. In addition, growth of *C. albicans* from the luminal to the abluminal compartment by intercalation between intact endothelial cells was rarely observed. Thus, these results suggest that the primary route of traversal of the endothelial monolayer, at least initially with undamaged endothelium, consists of the phagocytosis of the yeast form of *C. albicans* by endothelial cells. In agreement, heat-killed yeast forms were found to be efficiently phagocytosed by human vascular endothelial cells (8, 26). However, it cannot be excluded that in vivo the anchoring of hyphae or pseudohyphae in endothelial cells (26) helps to prevent physical detachment of fungi by the laminar flow of blood. Also, the damage of the endothelial cell layer by *C. albicans* (see below) or other factors (27, 30) may lead to endothelial traversal routes other than phagocytosis. The mechanism of exit of *C. albicans* on the abluminal side of endothelial cells is not known. By electron microscopy, we rarely caught fungi exiting from endothelial cells in our experimental system; however, in all cases, such fungi had formed true hyphae. In a previous study, even dead yeast forms were seen to exit from endothelial cells (8). It has been reported earlier that *C. albicans* is able to evade macrophages by hyphal growth (1). Thus, it appears plausible that hyphal formation may not be absolutely required but may accelerate the process of exiting from endothelial cells. Once fungi have left endothelial cells on the abluminal side, traversal of the extracellular matrix must ensue before infiltration of tissue parenchyma. The extracellular matrix may be exposed and become a focus of adhesion for *C. albicans* in case of endothelial contraction and/or damage (20). Fungal receptors for fibronectin and laminin are known (12, 23, 25), and the action of proteases (14) may contribute to the pene-

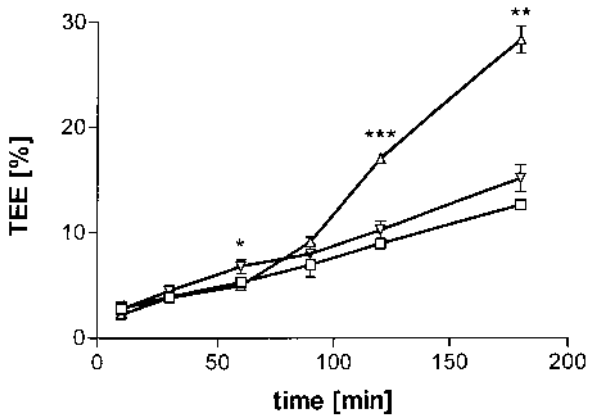

FIG. 5. Influence of *C. albicans* on the integrity of endothelium monolayer. The luminal side of a BAEC monolayer was preincubated with 1.0×10^6 cells of *C. albicans* ATCC 10261 (Δ) or strain hOG301 (∇) per ml for 6 h. Subsequently, 10 μM FD40 was added to the luminal compartment to determine its TEE. As a control, TEE values without fungal cells were determined (□). Experiments were carried out with three different strains of BAEC in 10 separate assays (mean values ± standard deviations). *P* values were calculated by using the *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

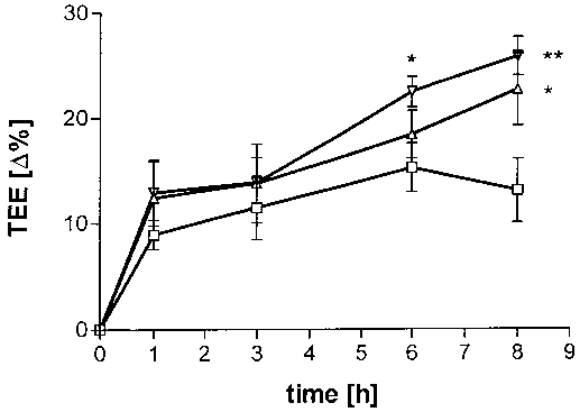

FIG. 7. Effect of glucose on TEE. TEE of FD40 across a BAEC monolayer pretreated with 1.0×10^6 *C. albicans* ATCC 10261 cells per ml was determined as for Fig. 5. The glucose concentrations were 0 (□), 5.5 (Δ), and 22 (∇) mM. Data are shown as the change in percent TEE, calculated as the difference between TEE values in the presence of *C. albicans* and TEE values in the absence of *C. albicans*. Experiments were carried out with three different strains of BAEC in 10 separate observations (means ± standard deviations). *P* values were calculated by using the *t* test: *, $P < 0.05$; **, $P < 0.01$.

tration of the extracellular matrix. It should be considered that a different composition of the extracellular matrix in diabetics (3) may also lead to altered penetration characteristics. In the described experimental system, an extracellular matrix is expected to be present below the endothelial cell layer (20) and to contribute to the overall characteristic of *C. albicans* migration from the luminal to the abluminal compartment of the endothelial monolayer. Thus, at least four essential aspects of the traversal of the endothel by *C. albicans* can be studied with our experimental system. Overall fungal migration was characterized by its dependence on the concentration of glucose and by the initial number of *C. albicans* cells in the luminal compartment. Conceivably, the described system may be applied to other endothelial cell types, such as human umbilical or porcine vascular endothelial cells that have been used in other studies (8, 19), as well as to epithelial cells.

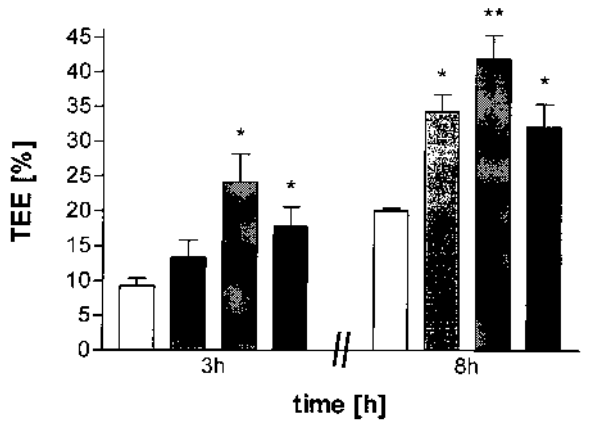

FIG. 6. Influence of *C. albicans* cell concentration on TEE. TEE of FD40 across a BAEC monolayer pretreated with different cell numbers of strain ATCC 10261 was determined as for Fig. 5. The fungal cell concentrations (cells per milliliter) used were 0.5×10^6 (light grey bars), 1.0×10^6 (dark grey bars), and 2.0×10^6 (black bars); in control experiments, no *C. albicans* cells were added (white bars). Data are the means ± standard deviations of 10 separate observations with three different strains of BAEC. *P* values were calculated by using the *t* test: *, $P < 0.05$; **, $P < 0.01$.

Damage of the endothelial monolayer by *C. albicans*. In an earlier study, coincubation with *C. albicans* was shown to cause rapid loss of the viability of porcine aortic endothelial cells (19). Recently, the damage to human vascular endothelial cells was quantitated by a chromium release assay, and it was demonstrated that phagocytosis of live but not dead *C. albicans* cells was required for damage to endothelial cells (8). Since the time course of endothelial cell injury coincided with hypha formation (7), it has been suggested that a cytotoxic substance secreted by hyphal cells (8) and/or, as discussed above, pseudohypha or hypha formation per se is required for efficient endothelial cell damage. In the experimental system described here, endothelial cell damage was detected by use of a labeled dextran, FD40, serving as a diffusion marker for TEE (32). Although the first *C. albicans* cells were found to appear after about 18 h, a significant increase in permeability to FD40 was already observed after 7 to 8 h. This result indicates that even at a time at which fungi are present within phagocytotic vacuoles, endothelial cell damage occurs. Conceivably, in the first phase, *C. albicans* infection causes cells to contract, allowing diffusion of FD40; at a later stage, endothelial cells may lyse completely and facilitate migration of whole fungi. Because damage of the endothelial monolayer requires a relatively long-term coincubation with *C. albicans*, it appears that internalization of live fungal cells and not mere contact with components in the fungal cell surface during adhesion is required. In agreement, isolated cell walls or membranes of *C. albicans* were not able to alter the permeability characteristics of the endothelial monolayer. As expected from the transendothelial migration characteristics, damage to endothelial cells as measured by diffusion of FD40 was found to depend on the glucose concentration, as well as the cell numbers of the fungal pathogen.

Migration characteristics of *C. albicans* mutants. We tested two *C. albicans* mutants in the migration assay, which were constructed by gene disruption (SGY-243) or by conventional mutagenesis (hOG301). Mutant SGY-243 does not grow or undergo morphogenesis in the absence of added uridine (17); its virulence is significantly reduced in the mouse model (18). In the migration system, strain SGY-243 was able to adhere to endothelial cells and to migrate to the abluminal compartment

like the wild type if uridine was added; in the absence of uridine, however, migration was reduced significantly. This result suggests that transendothelial migration of *C. albicans*, on the part of the fungus, can be a passive process (not excluding metabolic activity) but may become significantly enhanced by growth and/or morphogenesis. Mutant hOG301 produces pseudohyphae constitutively (13); virulence of hOG301 in the mouse model is reduced (28). Although hOG301 grew during the incubation in the luminal compartment, it did not adhere to endothelial cells but formed large aggregates. hOG301 was unable to reach the abluminal compartment during the assay, even in the absence of an endothelial monolayer, suggesting that aggregate formation did not allow passage through the pores in the filter support. In agreement with this behavior, unlike the parental wild-type strain, hOG301 did not increase the permeability of the endothelial monolayer. Thus, the observed inability to adhere to and damage endothelial cells is a likely reason for the reduced virulence of hOG301 in vivo. However, it should be considered that hOG301 arose by chemical mutagenesis, which may have induced multiple mutations, which may affect adherence, for example. Generally, the results obtained with the *C. albicans* mutants demonstrate that our transendothelial migration assay may be valuable in comparing and predicting virulence characteristics of *C. albicans* strains.

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