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Candida albicans Stimulates Cytokine Production and Leukocyte Adhesion Molecule Expression by Endothelial Cells

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Endothelial cells have the potential to influence significantly the host immune response to blood-borne microbial pathogens, such as *Candida albicans*. We investigated the ability of this organism to stimulate endothelial cell responses relevant to host defense in vitro. Infection with *C. albicans* induced endothelial cells to express mRNAs encoding E-selectin, intercellular adhesion molecule 1, vascular cell adhesion molecule 1, interleukin 6, interleukin 8, monocyte chemoattractant protein 1, and inducible cyclooxygenase (cox2). All three leukocyte adhesion molecule proteins were expressed on the surfaces of the endothelial cells after 8 h of exposure to *C. albicans*. An increase in secretion of all three cytokines was found after 12 h of infection. Cytochalasin D inhibited accumulation of the endothelial cell cytokine and leukocyte adhesion molecule mRNAs in response to *C. albicans*, suggesting that endothelial cell phagocytosis of the organism is required to induce this response. Live *Candida tropicalis*, *Candida glabrata*, a nongerminating strain of *C. albicans*, and killed *C. albicans* did not stimulate the expression of any of the cytokine or leukocyte adhesion molecule mRNAs. These findings indicate that a factor associated with live, germinating *C. albicans* is required for induction of endothelial cell mRNA expression. Furthermore, since endothelial cells phagocytize killed *C. albicans*, phagocytosis is likely necessary but not sufficient for this organism to stimulate mRNA accumulation. In conclusion, the secretion of proinflammatory cytokines and expression of leukocyte adhesion molecules by endothelial cells in response to *C. albicans* could enhance the host defense against this organism by contributing to the recruitment of activated leukocytes to sites of intravascular infection.

The nosocomial pathogen *Candida albicans* disseminates hematogenously in certain immunocompromised hosts and causes widespread microabscesses in multiple organs. During the process of hematogenous dissemination, the organism likely adheres to and then penetrates the endothelial cell lining of blood vessels to invade the deep tissues. Thus, endothelial cells are likely among the first host cells to interact with this organism.

Infections caused by different microbial pathogens elicit distinct patterns of inflammatory response. For example, microabscesses caused by *C. albicans* are characterized by suppurative granulomas composed of multinucleated giant cells, histiocytic macrophages, and neutrophils (14, 18, 24). In contrast, the inflammatory infiltrates elicited by *Staphylococcus aureus* are composed predominantly of neutrophils (52). The mechanisms responsible for these differences in host response are incompletely understood, but these differences are likely due in part to variations in the profile of cytokines and leukocyte adhesion molecules that are induced by the different microorganisms. Endothelial cells are known to synthesize a variety of immunomodulatory factors, including leukocyte adhesion molecules (48), cytokines (29), and eicosanoids (35). Therefore, it is probable that these cells play a significant role in determining the magnitude and profile of the host inflammatory response to hematogenously disseminated microbial pathogens.

Previously, we found that *C. albicans*, but not other species of *Candida*, stimulates endothelial cells to synthesize and release prostaglandins, mainly prostacyclin, in vitro (15, 16). The present study was conducted to determine further the potential influence of endothelial cells on the host inflammatory response to *C. albicans*. We investigated the effects of this organism on the expression of factors that are likely to be important in regulating the local inflammatory response to this organism while it is within the intravascular compartment. We found that live, germinating *C. albicans* stimulated endothelial cells to synthesize multiple proinflammatory factors in vitro. These factors included E-selectin, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1). This organism also induced the accumulation of mRNA for *cox2*, the inducible cyclooxygenase gene. The expression of these factors by endothelial cells in vivo could influence the host immune response to this organism during vascular invasion.

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MATERIALS AND METHODS

Organisms. *C. albicans* 36082 (a clinical isolate) was obtained from the American Type Culture Collection (Rockville, Md.). *C. albicans* SC5314 was graciously supplied by William Fonzi (George Washington University School of Medicine). A germination-deficient strain of *C. albicans*, V6, was generously provided by Helen Buckley (Temple University School of Medicine) (7). *Candida tropicalis* 4243 and *Candida glabrata* 31028 were clinical isolates obtained from the microbiology laboratory at Harbor-UCLA Medical Center. The organisms were grown and harvested by following a minor modification of our previously described methods (16). Briefly, the organisms were grown overnight in yeast nitrogen base broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose on a rotating drum at 25°C. The blastospores were harvested by centrifugation and washed twice in Dulbecco's phosphate-buffered saline (PBS) (Irvine

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Scientific, Santa Ana, Calif.). The organisms were counted in a hemacytometer and adjusted to the desired concentration in either Hank's balanced salt solution (HBSS) or RPMI 1640 medium (both from Irvine Scientific), depending on the experiment.

To obtain killed, germinated organisms, 3×10^8 blastospores of *C. albicans* 36082 were incubated in 100 ml of RPMI 1640 medium on a rotary shaker at 37°C for 90 min and then killed by exposure to 20 mM sodium periodate for 30 min at room temperature (17, 39). Greater than 90% of the organisms produced germ tubes prior to incubation with sodium periodate. The dead organisms were washed extensively in PBS, resuspended in HBSS, and enumerated as described above. An aliquot of the organisms was inoculated onto Sabouraud dextrose agar (Difco) to confirm that all of the organisms had been killed.

Endothelial cells. Endothelial cells were harvested from human umbilical veins by the method of Jaffe et al. (25). The cells were grown in M-199 (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Inc., Calabasas, Calif.), 10% defined bovine calf serum (Hyclone, Logan, Utah), and 2 mM L-glutamine with penicillin and streptomycin (Irvine Scientific). Neither heparin nor exogenous growth factors were added to the endothelial cell growth medium (15). All experiments were performed with second- to fourth-passage endothelial cells.

Endothelial cell damage. The extent of endothelial cell injury caused by the different stimuli was determined by chromium release assay as described previously (16, 17). Briefly, endothelial cells were grown in 24-well tissue culture plates (Falcon, Lincoln Park, N.J.) coated with 0.2% gelatin and incubated in M-199 containing $Na_2{}^{51}CrO_4$ (6 μCi per well; ICN Biomedicals, Irvine, Calif.) overnight. The following day, the unincorporated tracer was removed by extensive rinsing with HBSS, and 1 ml of M-199 was added to each well. The endothelial cells were infected by adding 1.3×10^5 organisms in 50 μl of HBSS so that the ratio of organisms to endothelial cells was 4:3. Next, the cells were incubated for selected time intervals at 37°C in 5% CO_2. At the end of incubation, half of the medium was gently aspirated from each well, and then 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 amounts of ^{51}Cr in the medium and the cell lysates were determined with a gamma counter. Control wells, which contained medium alone, were processed in parallel to measure the spontaneous release of ^{51}Cr. After correcting for differences in the incorporation of ^{51}Cr in each well, the specific release of ^{51}Cr was calculated by the following formula: [(2 × experimental release) − (2 × spontaneous release)]/[(total incorporation − (2 × spontaneous release)].

RNA extraction and Northern (RNA) blotting. Endothelial cells were grown to confluency in 75-cm^2 tissue culture flasks (Falcon) coated with 0.2% gelatin. The day before the experiment, the culture medium in each flask was replaced with 15 ml of fresh medium. The next day, 200 μl of HBSS containing either 5×10^6 or 7×10^4 organisms was added to the endothelial cells and the cells were incubated at 37°C in 5% CO_2. A similar volume of buffer or tumor necrosis factor alpha (TNF-α) (Sigma Chemical Company, St. Louis, Mo.) was added to control flasks of endothelial cells. The final concentration of TNF-α was 1 IU/ml.

In some experiments, to determine the effects of inhibiting endothelial cell phagocytosis of *C. albicans* on gene expression, cytochalasin D (Sigma) was added to the medium at a final concentration of 0.2 μM immediately before the addition of *C. albicans* 36082. Control endothelial cells were exposed to an equal amount of the diluent (dimethyl sulfoxide; final concentration, 0.01%) prior to inoculation. In these experiments, the incubation time was 6 h. These conditions were chosen because in preliminary experiments it was determined that higher concentrations of and longer exposures to cytochalasin D caused significant endothelial cell injury, even in the absence of *C. albicans*. Previously, we determined that cytochalasin D has no effect on candidal germination and elongation (17).

At selected time points, the conditioned media were aspirated from the flasks and centrifuged at 1,500 × *g* and 4°C for 10 min, after which the supernatants were stored at −70°C for analysis of cytokine content (see below). Next, the endothelial cells were rinsed with 10 ml of warm HBSS, and then the endothelial cell RNA was extracted with guanidium-phenol-chloroform by the method of Chomczynski and Sacchi (9). Endothelial cell gene expression was assessed by Northern blotting using standard techniques (40). Briefly, 10 μg of total RNA was electrophoresed through 1.2% agarose-formaldehyde gels and then transferred to nylon membranes (Micron Separation, Inc., Westboro, Mass.). The membranes were hybridized with cDNA probes labeled with [^{32}P]dCTP by the random primer method (NEBlot; New England Biolabs, Beverly, Mass.). The probes that were used and their sources are listed in Table 1. Gene expression was detected by autoradiography and quantified by densitometry. The results were normalized to the expression of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to correct for any differences in the amount of RNA added to each lane.

In preliminary experiments, we determined that the RNA extraction technique extracted RNA only from the endothelial cells and not from *C. albicans*. Moreover, RNA from *C. albicans* did not hybridize with any of the human cDNA probes.

Detection of cytokines in conditioned media. To determine the effects of *C. albicans* on endothelial cell secretion of the IL-6, IL-8, and MCP-1 proteins, conditioned media from two different types of experiments were analyzed. The first set of conditioned media was obtained prior to extraction of RNA from the endothelial cells. These media were from endothelial cells that were grown in 75-cm^2 flasks and exposed to 5×10^6 live blastospores of *C. albicans* 36082, 1 IU of TNF-α per ml, or buffer alone for 8 h. Conditioned media obtained from six independent experiments were analyzed. A second set of conditioned media was collected from endothelial cells grown in six-well tissue culture plates (Falcon) and exposed to the different stimuli for 12 h. These experiments were performed in duplicate on three separate occasions. In these experiments, the endothelial cells were exposed to 6.4×10^5 *C. albicans* 36082 cells in 2 ml of medium. These conditions were chosen so that the ratio of organisms to endothelial cells and volume of medium per 10^5 endothelial cells were identical to those used in the 75-cm^2 flask experiments. The concentrations of IL-6, IL-8, and MCP-1 in the conditioned media were determined by commercial enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, Minn.).

TABLE 1. Human cDNA probes used for Northern blotting

Gene	Source	Reference
cox2	Timothy Hla, American Red Cross, Bethesda, Md.	23
E-selectin	Laurelee Osborn, Biogen, Inc., Cambridge, Mass.	21
GAPDH	American Type Culture Collection (57090)[a]	50
ICAM-1	Mark Zukowski, Amgen, Inc., Boulder, Colo.	44
IL-6	American Type Culture Collection (68636)[a]	None
IL-8	Kouji Matsushima, National Cancer Institute, Fredrick, Md.	30
MCP-1	Judith Berliner, UCLA School of Medicine, Los Angeles, Calif.	10
VCAM-1	Laurelee Osborn, Biogen, Inc., Cambridge, Mass.	34

[a] Catalog number is given in parentheses.

Indirect immunofluorescence. Indirect immunofluorescence was used to detect the expression of leukocyte adhesion molecules on the endothelial cells. The endothelial cells were grown to confluency on 12-mm-diameter circular glass coverslips coated with human fibronectin (Becton Dickinson, Bedford, Mass.). Fibronectin was used to obtain better adherence and growth of the endothelial cells on the glass coverslips. The endothelial cells were infected with 5×10^4 blastospores of *C. albicans* 36082 and incubated for 8 h at 37°C in 5% CO_2. Next the cells were rinsed with HBSS containing 2% (vol/vol) FBS (HBSS-FBS). The coverslips were inverted onto 50 μl of HBSS-FBS containing 5 μg of primary antibody per ml. In different experiments, the primary antibodies were monoclonal antibodies directed against E-selectin (generously provided by Michael Bevilacqua, Amgen, Inc., Boulder, Colo.), ICAM-1 (Immunotech, Inc., Westbrook, Maine), or VCAM-1 (Immunotech). After incubation at 4°C for 1 h, the coverslips were rinsed in ice-cold HBSS-FBS, fixed in 0.5% (wt/vol) paraformaldehyde in PBS for 15 min, and blocked in PBS containing 2% (vol/vol) FBS (PBS-FBS) overnight. The following day, the coverslips were inverted onto 50 μl of fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Sigma) for 1 h at 4°C, rinsed extensively in PBS-FBS, and then mounted, inverted, on glass slides. The cells were viewed with a Zeiss Axiovert 10 microscope (Carl Zeiss, Inc., Thornwood, N.J.) equipped for epifluorescence and photographed with Tmax 400 film (Eastman Kodak, Rochester, N.Y.).

Endotoxin testing. The amount of endotoxin in the endothelial cell growth medium and the yeast nitrogen base broth was measured by a chromogenic limulus amebocyte lysate test (BioWhittaker, Inc., Walkersville, Md.). The concentration of endotoxin in each culture medium was less than 0.1 IU/ml.

Statistical analysis. The effects of the various stimuli on endothelial cell injury and cytokine secretion were analyzed by using the Kruskal-Wallis test with the Bonferroni correction for multiple comparisons. *P* values of ≤0.05 were considered to be significant.

RESULTS

Exposure to *C. albicans* stimulated cytokine and leukocyte adhesion molecule gene expression in endothelial cells. The live blastospores of *C. albicans* 36082 began germinating 1 h after being added to the endothelial cells. After 4 h of infection, most of the endothelial cells were in contact with one or more candidal germ tubes. At this time, the endothelial cells began expressing mRNAs encoding the leukocyte adhesion molecules E-selectin, ICAM-1, and VCAM-1 (Fig. 1A). Exposure to *C. albicans* also stimulated endothelial cells to accumulate mRNAs for IL-6, IL-8, and MCP-1, as well as *cox2* (Fig. 1B). The amounts of these mRNAs rose steadily as the duration of infection increased. In contrast, endothelial cells ex-

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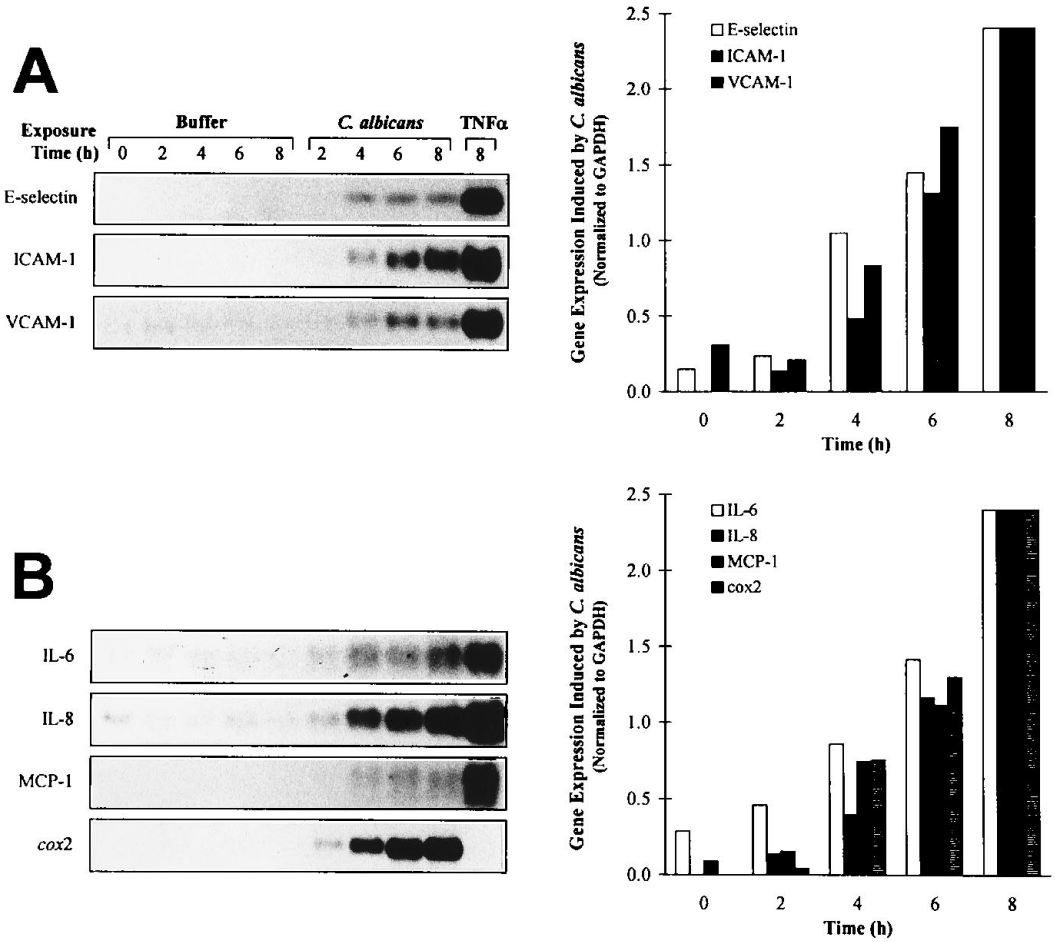

FIG. 1. The expression of mRNAs encoding leukocyte adhesion molecules (A) as well as cytokines and *cox2* (B) following infection of endothelial cells with *C. albicans*. Accumulation of the indicated mRNAs was assessed by Northern blotting. The photographs on the left are of the actual autoradiograms. The bar graphs on the right show mRNA expression as quantified by densitometry and normalized to the expression of GAPDH. The indicated symbols from top to bottom correspond to the bars from left to right. The results are representative of one of three individual experiments.

posed to media alone contained low to undetectable levels of the leukocyte adhesion molecule and cytokine mRNAs at the same time points. The rate of mRNA accumulation in response to *C. albicans* appeared to be similar for all genes tested. Infection with *C. albicans* had no effect on the expression of mRNA from the constitutively expressed GAPDH gene (data not shown).

The magnitude of the endothelial cell response to *C. albicans* was different from that induced by TNF-α. Infection with *C. albicans* for 8 h induced a greater accumulation of *cox2* mRNA than was stimulated by TNF-α. However, TNF-α consistently induced greater expression of the cytokine and leukocyte adhesion molecule mRNAs than did *C. albicans.*

The above experiments were performed with a 1.3:1 ratio of *C. albicans* to endothelial cells. In other experiments, we infected the endothelial cells with 70-fold fewer organisms to determine if prolonged exposure to a smaller inoculum altered the pattern of mRNA expression. Reducing the number of organisms slowed the kinetics of endothelial cell stimulation. With this inoculum, there was minimal stimulation of mRNA accumulation at 12 h. However, expression of the cytokine and leukocyte adhesion molecule mRNAs was induced more strongly after 24 h of infection. Nevertheless, the magnitude of this stimulation was less than that induced by even 6 h of exposure to the higher inoculum of *C. albicans* (data not shown).

Infection with *C. albicans* induced the surface expression of leukocyte adhesion molecules on endothelial cells. To determine if *C. albicans* stimulates endothelial cells to express leukocyte adhesion molecules on their surfaces, the endothelial cells were infected for 8 h at a 1:2 ratio of organisms to endothelial cells. The lower inoculum was used in these experiments so that we could better observe whether direct contact with an organism was required to stimulate an individual endothelial cell.

Minimal expression of E-selectin and VCAM-1 was observed on the surfaces of unstimulated endothelial cells (Fig. 2A and G). However, these cells expressed low levels of ICAM-1 (Fig. 2D). Consistent with the results of the Northern blotting experiments, we found that exposure to *C. albicans* stimulated endothelial cells to express E-selectin, ICAM-1, and VCAM-1 proteins (Fig. 2B, E, and H, respectively). There was marked cell-to-cell variation in the degree of leukocyte adhesion molecule expression induced by *C. albicans*. Some endothelial cells exhibited strong expression, while others showed only basal levels of leukocyte adhesion molecule expression. The majority, but not all, of the stimulated endothelial cells were in contact with one or more candidal germ tubes. Also, on some endothelial cells, the leukocyte adhesion molecules appeared to be expressed most strongly in the region immediately adjacent to the organisms, so that the germ tubes were outlined

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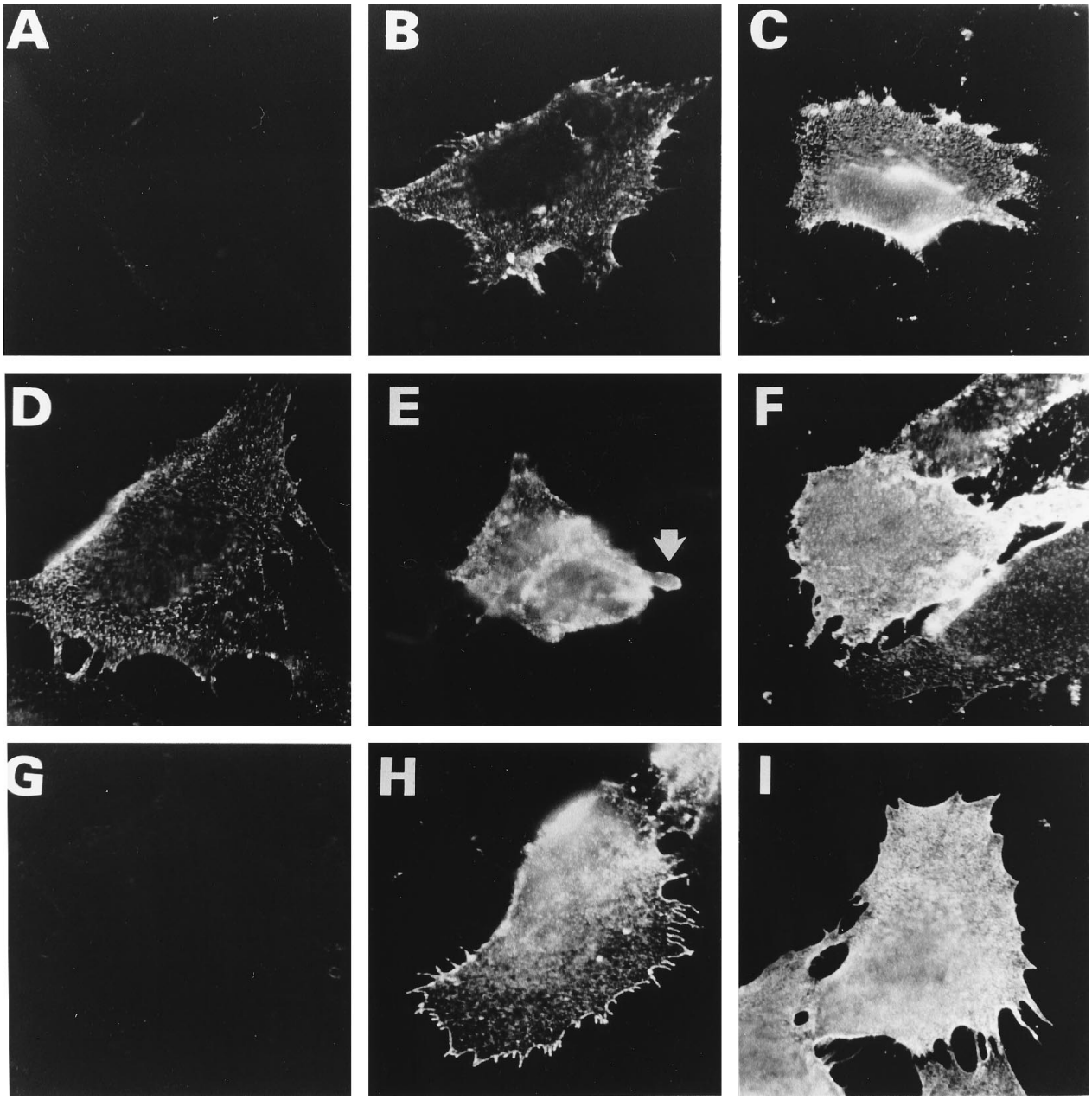

FIG. 2. Expression of leukocyte adhesion molecules on the surfaces of endothelial cells after exposure to *C. albicans*. Endothelial cells were incubated with buffer (A, D, G), live blastospores of *C. albicans* 36082 (B, E, H), or TNF-α (C, F, I) for 8 h. The surface expression of E-selectin (A to C), ICAM-1 (D to F), and VCAM-1 (G to I) was detected by indirect immunofluorescence. The arrow in panel E indicates ICAM-1 that has accumulated around a candidal germ tube. The results are representative of one of three separate experiments.

by leukocyte adhesion molecules (Fig. 2E). The candidal germ tubes did not appear to express any leukocyte adhesion molecules themselves, because the antibodies only bound to organisms that were in direct contact with activated endothelial cells.

In contrast to the infected endothelial cells, the cells stimulated with TNF-α exhibited greater surface expression of leukocyte adhesion molecules and there was less cell-to-cell variability in the level of expression (Fig. 2C, F, and I). Furthermore, the leukocyte adhesion molecules were expressed in a uniform speckled pattern on the surfaces of individual cells that were exposed to TNF-α.

***C. albicans* stimulated endothelial cells to secrete IL-6, IL-8, and MCP-1 proteins.** The ability of *C. albicans* to stimulate endothelial cells to secrete IL-6, IL-8, and MCP-1 was examined. First, we tested the conditioned media that were collected prior to extraction of the endothelial cell RNA. After 8 h of exposure to the organism, the concentrations of IL-6, IL-8, and MCP-1 in the conditioned media were increased only slightly above basal levels (Table 2). This lack of cytokine secretion contrasted with the marked accumulation of the corresponding mRNAs in the endothelial cells at this time point (Fig. 1). In addition, these endothelial cells responded to TNF-α with a significant increase in secretion of all three cytokines.

Next, we exposed the endothelial cells to *C. albicans* for 12 h and found that the organisms stimulated a significant increase in the concentration of each of the three cytokines in media

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TABLE 2. Cytokines released by endothelial cells in response to *C. albicans* and TNF-α

Stimulus	Percent increase in concentration of cytokine[a]					
	IL-6		IL-8		MCP-1	
	After 8 h	After 12 h	After 8 h	After 12 h	After 8 h	After 12 h
C. albicans	9 (5–13)[b]	25 (13–43)[b]	−5 (−4–23)	56 (55–67)[b]	4 (2–6)[c]	15 (14–37)[b]
TNF-α	59 (56–92)[c]	114 (100–128)[b]	160 (147–191)[b]	356 (328–411)[b]	118 (76–136)[b]	173 (164–188)[b]

[a] Concentrations are in comparison to those in media from endothelial cells exposed to buffer alone. The values are medians of data obtained in three to six individual experiments, and the numbers in parentheses are the first to third quartile ranges. Median unstimulated cytokine concentrations (determined by ELISA) were as follows: after 8 h, IL-6, 1.7 ng/ml; IL-8, 52 ng/ml; and MCP-1, 28 ng/ml; after 12 h, IL-6, 1.1 ng/ml; IL-8, 29 ng/ml; and MCP-1, 28 ng/ml.
[b] $P \leq 0.008$ versus unstimulated endothelial cells.
[c] $P < 0.03$ versus unstimulated endothelial cells.

collected at this time point (Table 2). Of the three cytokines, IL-8 was secreted in the highest amounts in response to *C. albicans*. As expected (53), we observed a marked variability in the amount of each cytokine that was secreted by endothelial cells from different umbilical cords. However, 12 h of exposure to *C. albicans* consistently enhanced the secretion of all three cytokines by the infected endothelial cells.

Cytochalasin D inhibited endothelial cell mRNA expression induced by *C. albicans* but not that induced by TNF-α. Cytochalasin D was used to investigate whether endothelial cell phagocytosis of *C. albicans* was necessary for endothelial cell stimulation to occur. Adding this agent to the endothelial cells just prior to addition of the organisms markedly reduced the expression of the leukocyte adhesion molecule and cytokine mRNAs in response to *C. albicans* (Fig. 3). However, the accumulation of *cox2* mRNA was incompletely suppressed by this agent. A likely explanation for this partial inhibition of *cox2* mRNA expression was our finding that cytochalasin D by itself moderately induced the accumulation of *cox2* mRNA. Cytochalasin D had no effect on endothelial cell mRNA expression induced by TNF-α, nor did it affect the constitutive expression of GAPDH mRNA. Therefore, it is likely that endothelial cell phagocytosis of *C. albicans* is required for the organism to stimulate mRNA expression in these cells.

Only live, germinated *C. albicans* stimulated endothelial cell mRNA expression. To evaluate whether factors specific to *C. albicans* were required for the stimulation of endothelial cells to occur, the cells were infected with a germination-deficient strain of *C. albicans*, V6, as well as with *C. tropicalis* and *C. glabrata*. The endothelial cells were also exposed to a second wild-type strain of *C. albicans*, SC5314, and to periodate-killed germ tubes of *C. albicans* 36082. We found previously that these killed organisms are phagocytized by endothelial cells (17, 39). As shown in Fig. 4, only live, germinated *C. albicans* induced the accumulation of mRNA from *cox2* and the genes encoding cytokines and leukocyte adhesion molecules. In contrast, none of the other organisms consistently altered the level

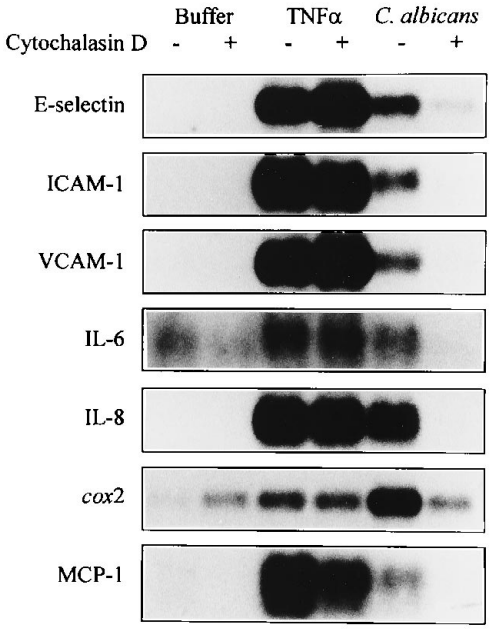

FIG. 3. Effects of cytochalasin D on endothelial cell mRNA expression. Endothelial cells were exposed for 6 h to buffer alone, TNF-α, or *C. albicans* 36082 in the absence (−) or presence (+) of 0.2 μM cytochalasin D. The accumulations of the indicated mRNAs were determined by Northern blotting. The results are representative of three separate experiments.

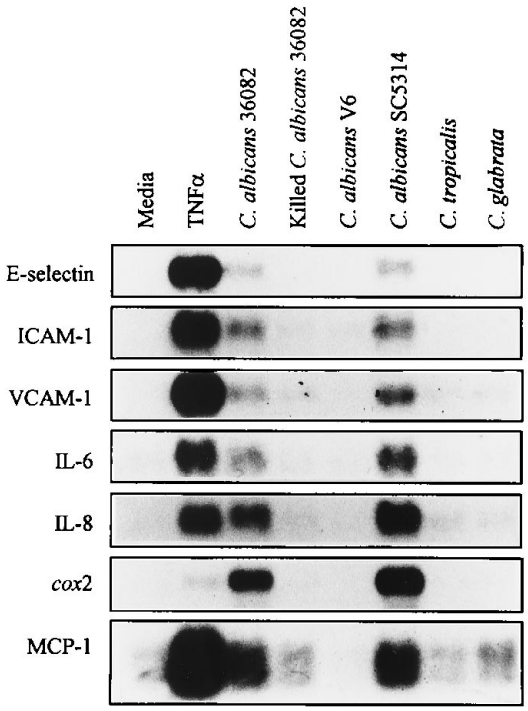

FIG. 4. Endothelial cell mRNA expression in response to live *Candida* species, killed *C. albicans*, and TNF-α. Endothelial cells were incubated with media alone or with the indicated stimuli for 8 h. The accumulation of cytokine and leukocyte adhesion molecule mRNAs was measured by Northern blotting. The autoradiographs are representative results from one of three experiments.

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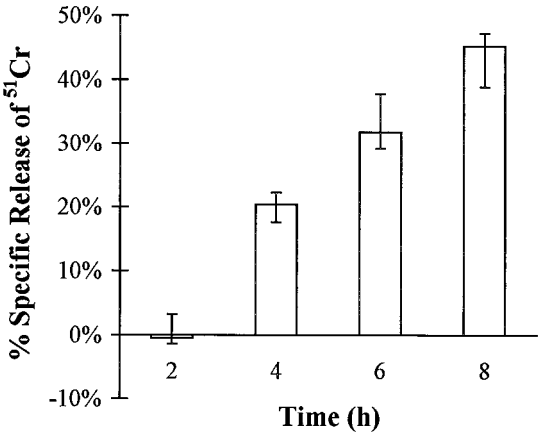

FIG. 5. Time course of endothelial cell injury caused by *C. albicans*. Endothelial cells loaded with ^{51}Cr were exposed to live blastospores of *C. albicans* 36082 for the indicated time periods. The extent of *Candida*-induced endothelial cell injury was measured by the specific release of ^{51}Cr. The results are the medians of three separate experiments, each performed in triplicate. The error bars indicate the first to third quartiles.

of mRNA expression from any of these genes. Therefore, a factor associated with the germination of *C. albicans* is likely required for the organism to stimulate endothelial cell mRNA expression. Furthermore, since dead, germinated *C. albicans* were phagocytized but did not induce mRNA accumulation, phagocytosis appears to be necessary but not sufficient for this organism to stimulate mRNA expression in endothelial cells.

Candidal induction of endothelial cell mRNA expression and endothelial cell injury were closely associated. Previously, we had found that the extent of endothelial cell injury caused by *C. albicans* was closely associated with the amount of prostacyclin that is synthesized by endothelial cells in response to this organism (16). Therefore, we compared the time course of endothelial cell injury caused by *C. albicans* with the time course of mRNA accumulation. As shown in Fig. 5, candidal damage to the endothelial cells was detectable after 4 h of infection, and it increased with further exposure to the organisms. This time course of injury roughly paralleled the increase in endothelial cell mRNA expression at the same time points (Fig. 1).

The effect of cytochalasin D on endothelial cell injury by *C. albicans* also corresponded to its effect on *Candida*-induced mRNA expression. When used under conditions similar to those used in the gene expression experiments, cytochalasin D reduced endothelial cell injury by *C. albicans* from a median of 39% (first quartile, 26%; third quartile, 42%) to 5% (8 and 0%, respectively) ($P < 0.001$). Similarly, only those organisms that stimulated mRNA accumulation in endothelial cells caused endothelial cell injury. After 8 h of infection, the live, wild-type strains of *C. albicans*, 36082 and SC5314, induced median specific releases of ^{51}Cr of 46 and 45%, respectively (interquartile range, 42 to 52% for both strains), whereas *C. tropicalis*, *C. glabrata*, *C. albicans* V6, and periodate-killed *C. albicans* 36082 did not cause any detectable endothelial cell injury. Thus, in response to *C. albicans*, endothelial cell mRNA expression was closely associated with endothelial cell injury. This association was not observed with TNF-α. At the concentration used in the experiments, this cytokine did not cause significant endothelial cell damage.

DISCUSSION

Infection with *C. albicans* in vitro stimulated the synthesis of cytokines and leukocyte adhesion molecules by endothelial cells. The local production of these immunomodulatory factors likely contributes to the development of the mixed acute and chronic inflammatory infiltrate seen at sites of candidal infection in vivo. For example, the release of IL-8 and the surface expression of E-selectin and ICAM-1 likely mediate the recruitment of neutrophils to these areas (1, 45, 55). Furthermore, IL-8 has been shown to enhance the ability of neutrophils to kill *C. albicans* in vitro (12). Elevated levels of this cytokine have been found in neutropenic patients with invasive infections caused by *C. albicans* (51). However, the cellular sources of this cytokine during such infections have not yet been determined. In addition, it is not known currently which leukocyte adhesion molecules are expressed during the evolution of hematogenously disseminated candidiasis in animal models or in humans.

We also found that *C. albicans* induced endothelial cells to synthesize and release IL-6. In addition to stimulating B-cell activity (22) and inducing the production of acute-phase reactants (8), this cytokine enhances the priming effects of TNF-α and platelet-activating factor on neutrophil function (2, 33). The critical role of IL-6 in the host defense against intracellular pathogens has been demonstrated in IL-6-deficient mice. Infection with *Listeria monocytogenes* causes high mortality in these mice, and there is a striking absence of neutrophilia in the peripheral blood of the IL-6-deficient animals (11). It is known that the serum concentration of this cytokine is elevated in mice with hematogenously disseminated candidiasis (49). Moreover, the serum level of IL-6 is significantly higher when mice are made neutropenic, suggesting that neutrophils may have a negative-feedback effect on the production of this cytokine in vivo.

In addition to producing substances that recruit and activate neutrophils, endothelial cells infected with *C. albicans* expressed VCAM-1 and secreted MCP-1. These factors, along with ICAM-1, may contribute to the accumulation of mononuclear cells at sites of candidal infection (34, 38, 55). Although neutrophils are considered to be the most important phagocyte in the host defense against hematogenously disseminated candidiasis, mononuclear cells may also contribute to the defense against this type of infection, especially when there is neutropenia (26, 27).

Previously we have reported that in vitro infection with *C. albicans* stimulates endothelial cells to secrete prostaglandins (16). It was found that this enhanced prostacyclin secretion is mediated by two processes: (i) increased mobilization of endothelial cell arachidonic acid and (ii) de novo synthesis of cyclooxygenase (15). Our current finding that *C. albicans* stimulates the accumulation of *cox2* mRNA provides a mechanism to explain how the latter process occurs. Prostacyclin is known to inhibit neutrophil adherence and superoxide release (19, 57). Thus, the secretion of this prostaglandin in response to *C. albicans* may serve to modulate the local inflammatory response and prevent leukocyte-mediated damage of host cells.

Microorganisms other than *C. albicans* have been found to stimulate the production of immunomodulatory substances by endothelial cells in vitro. For example, infection with *Rickettsia rickettsii* stimulates endothelial cells to express E-selectin (47), and *Treponema pallidum* induces the expression of ICAM-1 on the surfaces of these cells (37). *Borrelia burgdorferi* has been shown to induce endothelial cell expression of E-selectin, VCAM-1, and ICAM-1, both in human umbilical vein endothelial cells in vitro and in experimentally infected mice (42,

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43). However, the in vitro response to *B. burgdorferi* is different from that induced by *C. albicans*. Infection with *B. burgdorferi* stimulates maximal E-selectin expression at 4 h, and expression of ICAM-1 and VCAM-1 peaks at 12 h (43). In contrast, we found that *C. albicans* stimulated the accumulation of mRNAs for all three leukocyte adhesion molecules at the same rate, and mRNA accumulation continued to increase until at least 8 h after infection.

Although *C. albicans* stimulated the accumulation of the different mRNAs at roughly the same rate, there were distinct differences between the surface expression of the leukocyte adhesion molecule proteins and the secretion of the cytokines. All three leukocyte adhesion molecules were expressed strongly after 8 h of exposure to *C. albicans*. However, 12 h of infection was required for the organism to stimulate a significant increase in the concentrations of IL-6, IL-8, and MCP-1 proteins in the media. Although the increases in IL-6 and MCP-1 accumulation were statistically significant at 12 h, the magnitudes of the increases were low. One possible explanation for these findings is that *C. albicans* is a stronger stimulus for mRNA expression than it is for protein secretion. For example, the endothelial cells may be damaged sufficiently by *C. albicans* such that protein synthesis and/or secretion is inhibited even though increased levels of the cytokine mRNAs are present. Alternatively, proteases secreted by the organism (4, 54) may degrade the secreted cytokines and reduce their accumulation.

The finding that cytochalasin D inhibited the expression of cytokine and leukocyte adhesion molecule mRNAs in response to *C. albicans*, but not in response to TNF-α, is evidence that endothelial cell phagocytosis of the organism is required for endothelial cell stimulation to occur. On the basis of similar experiments, Yao et al. (56) concluded that the phagocytosis of intact *S. aureus* by endothelial cells is required for the induction of IL-6 mRNA accumulation in endothelial cells. Nevertheless, it is likely that *S. aureus* stimulates endothelial cells by a different mechanism than does *C. albicans*. We determined that killed *C. albicans* did not induce endothelial cell mRNA expression, even though such organisms are phagocytized by endothelial cells (17, 29). In contrast, endothelial cell expression of IL-6 mRNA is induced by dead *S. aureus* (56). Furthermore, purified capsular polysaccharides from *S. aureus* have been found to stimulate endothelial cells to secrete both IL-8 and IL-6 (46). Therefore, *S. aureus* may stimulate endothelial cells by a mechanism different from that of *C. albicans.*

Although candidal viability is necessary for the organisms to induce endothelial cell mRNA expression, it is not required for the stimulation of leukocytes. For example, exposure to heat-killed *C. albicans* stimulates neutrophils to secrete IL-8 (12) and monocytes to release IL-6 (36). This difference in response between endothelial cells and leukocytes is probably not due to differences in the method of killing the organism. Although we used periodate-killed *C. albicans* in the current experiments, we had found previously that heat-killed organisms did not induce endothelial cell prostacyclin synthesis (16), even though they were phagocytized by endothelial cells (unpublished data). Thus, it is highly likely that *C. albicans* stimulates endothelial cells by a different mechanism than it uses to stimulate neutrophils or monocytes.

In addition, the finding that killed organisms had no effect on endothelial cell gene expression provides substantial evidence that the endothelial cells were stimulated directly by live organisms rather than being stimulated indirectly by cytokines released from any contaminating leukocytes that may have been associated with the endothelial cells. Also, the consistent differences in the abilities of the various organisms to stimulate endothelial cell mRNA accumulation, combined with the results of the cytochalasin D experiments, make it highly unlikely that the observed pattern of endothelial cell stimulation was the result of endotoxin contamination.

In addition to requiring candidal viability, endothelial cell stimulation is likely dependent on one or more factors that are associated with candidal germination, because only organisms that germinated induced accumulation of the leukocyte adhesion molecule and cytokine mRNAs. It is known that transformation from the blastospore to the germinated form is accompanied by significant changes in the surface characteristics of *C. albicans* (6, 31, 32) and the differential expression of multiple genes (3, 41, 54). However, which of these changes enable the organism to stimulate endothelial cell gene expression is unknown at present.

A striking finding was that only conditions associated with candidal injury of endothelial cells stimulated endothelial cell mRNA accumulation. Thus, it is possible that endothelial cell injury is the stimulus for gene expression. Irrespective of the mechanism by which *C. albicans* stimulates endothelial cells, our finding that the organism induces endothelial cells to secrete proinflammatory cytokines and express leukocyte adhesion molecules suggests that endothelial cells may actively recruit leukocytes to areas of vascular invasion in vivo.

Other investigators have examined the effects of injurious stimuli on the expression of leukocyte adhesion molecules by endothelial cells. For example, low concentrations of hydrogen peroxide induce endothelial cells to express ICAM-1 but not E-selectin or VCAM-1 (5). Also, exposing endothelial cells to UV radiation stimulates them to express E-selectin and ICAM-1, without inducing VCAM-1 expression (20). Therefore, the endothelial cell responses to these stimuli are different from the response induced by *C. albicans*, which stimulated the expression of all three types of leukocyte adhesion molecules. It is currently unknown if different types of microbially induced injury stimulate differing profiles of endothelial cell responses.

One mechanism by which microbially induced cellular damage can lead to cellular activation has been reported by Eckmann et al. (13). They found that *Entamoeba histolytica* injures epithelial cells in vitro and causes them to release IL-1α into the medium. The IL-1α stimulates adjacent epithelial cells by a paracrine mechanism and induces them to synthesize cytokines, such as IL-8 and IL-6. A similar mechanism has been found to be operative in the stimulation of endothelial cells by *Rickettsia conorii* (28). It is possible that endothelial cell injury by *C. albicans* also results in the release of IL-1α. Such a paracrine mechanism would account for the close relationship between endothelial cell injury and induction of endothelial cell mRNA expression. In addition, it would explain why some endothelial cells that were not in direct contact with *C. albicans* were observed to express leukocyte adhesion molecules on their surfaces in the indirect-immunofluorescence experiments. Studies to examine whether IL-1α release mediates endothelial cell stimulation by *C. albicans* are currently in progress in our laboratory.

The capacity of endothelial cells to respond to *C. albicans* by expressing leukocyte adhesion molecules and secreting proinflammatory cytokines in vitro suggests that these cells may enhance the host defense against this organism in vivo. The expression of such factors by endothelial cells may augment the recruitment of activated leukocytes to sites of intravascular infection. In vivo, the response of endothelial cells to bloodborne microbial pathogens is likely influenced by cytokines and other immunomodulatory factors produced by neutrophils, monocytes, and lymphocytes. Determining the nature of the signals that are exchanged between these different cell types in

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response to fungal infection may lead to the development of novel endothelial-cell-based strategies to prevent or treat these infections.

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