Rhizopus oryzae Adheres to, Is Phagocytosed by, and Damages Endothelial Cells In Vitro

Ashraf S. Ibrahim,^{1,2*} Brad Spellberg,^{1,2} Valentina Avanesian,¹ Yue Fu,^{1,2}
and John E. Edwards, Jr.^{1,2}

Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance,¹ and
David Geffen School of Medicine at UCLA, Los Angeles,² California

Received 20 September 2004/Returned for modification 11 October 2004/Accepted 19 October 2004

Rhizopus oryzae is the most common cause of zygomycosis, a life-threatening infection that usually occurs in immunocompromised patients. A characteristic hallmark of zygomycosis is angioinvasion by the fungus, resulting in thrombosis and subsequent tissue necrosis. Interactions between *R. oryzae* and vascular endothelial cells are therefore likely of central importance to the organism's pathogenetic strategy. We studied the ability of *R. oryzae* to adhere to and damage human umbilical vein endothelial cells (HUVECs) in vitro. We report that *R. oryzae* spores and germ tubes adhere to HUVECs, whereas only spores adhere to subendothelial matrix proteins. Additionally, *R. oryzae* damages endothelial cells. This endothelial cell damage requires direct contact and subsequent phagocytosis of the fungus. Surprisingly, *R. oryzae* viability was not required for damage, but phagocytosis was required for dead *R. oryzae* to cause damage. These results elucidate the nature of *R. oryzae*-endothelial cell interactions, which are likely central to the angioinvasion and tissue necrosis seen during zygomycotic infections. The fact that dead *R. oryzae* damage human endothelial cells may, in part, explain the lack of efficacy of fungicidal agents during clinical disease.

Rhizopus oryzae is the organism most frequently isolated from patients with zygomycosis (6, 12), a highly destructive and lethal infection in immunocompromised hosts (2, 6, 9, 13). The standard therapy for invasive zygomycosis consists of reversal of the underlying predisposing factors, widespread surgical debridement, and aggressive antifungal medication (2, 6, 13). Unfortunately, despite disfiguring surgical debridement and aggressive therapy with amphotericin B, the overall mortality of zygomycosis remains >50% (13), and it approaches 100% in patients with disseminated disease (5). Clearly, new strategies to treat zygomycosis are urgently needed.

A hallmark of mucormycosis infections is the virtually uniform presence of extensive angioinvasion with resultant vessel thrombosis and tissue necrosis (2, 6, 9, 13). This angioinvasive character is associated with the ability of the organism to spread through viable tissue and to hematogenously disseminate to other target organs. Furthermore, since antifungal agents are carried to the site of infection in the vasculature, adequate blood supply is necessary to deliver these agents to *R. oryzae* in vivo. Therefore, ischemic necrosis as a result of *R. oryzae*-mediated angioinvasion is likely an important mechanism by which the fungus survives therapy with fungicidal agents, such as amphotericin B. For these reasons, damage of and penetration through endothelial cells lining blood vessels is likely a critical step in *R. oryzae*'s pathogenetic strategy.

We therefore studied the interaction between *R. oryzae* and endothelial cells. We find that damage to endothelial cells from *R. oryzae* is dependent upon its adherence to and phago-

cytosis by endothelial cells. Surprisingly, *R. oryzae* does not need to be viable to cause endothelial cell damage. These results have important implications in the pathogenesis of mucormycosis and may ultimately impact antifungal therapy of this extremely lethal infection.

MATERIALS AND METHODS

Organisms and culture conditions. Clinical isolates of Mucorales were obtained from the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio. These organisms included *R. oryzae* 99–880 (a brain isolate), 99–892 (a lung isolate), and 99–133 (a bone marrow isolate) and *Mucor* sp. strain 97–1083 (a blood isolate). *R. oryzae* HUMC02 was obtained from a patient with rhinocerebral mucormycosis treated at Harbor-UCLA Medical Center. The organisms were grown on potato dextrose agar (PDA) for 3 days at 37°C. The sporangiospores were collected in endotoxin-free phosphate-buffered saline (PBS), pH 7.4, (Irvine Scientific, Irvine, Calif.) containing 0.01% Tween 80 and washed once with PBS without Tween 80. Spores were sonicated for 5 s, using a Branson Sonifier 450 (output level 3; Branson Ultrasonics, Danbury, Conn.), counted with a hemacytometer, and adjusted to the desired concentration in RPMI 1640 with glutamine (Gibco, Grand Island, N.Y.).

To obtain germ tubes of *R. oryzae*, spores were harvested from PDA plates and processed as described above. An inoculum of 5×10^6 spores per ml was added to YPD medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% Bacto-peptone [Difco], 2% D-glucose) and shaken at 37°C for 4 to 5 h until small germ tubes (3 to 5 μ m) protruded from the cells as determined by microscopic examination. Because of their small size, pregerminated hyphae could be accurately quantified by counting on a hemacytometer, just like spores.

To examine the effect of viability on endothelial cell interactions, *R. oryzae* spores or germ tubes (5×10^5 /ml) were killed by heating in a water bath at 60°C for 1 h or suspension in 70% ethanol or 2.5% glutaraldehyde for 30 min. Cells were washed with PBS, and an aliquot of the organisms was inoculated onto PDA for overnight culture at 37°C to confirm that the fungi had been killed.

Saccharomyces cerevisiae CRY2 α (4) was a generous gift from Y. Wang (Institute of Molecular and Cellular Biology, Singapore). This strain was maintained on YPD medium until used in the adherence assay.

Preparation of endothelial cells. Human umbilical vein endothelial cells (HUVECs) were obtained by a modification of the method of Jaffe et al. (8). The cells were grown in M-199 medium containing 2 mM L-glutamine, penicillin, and streptomycin (all from Gibco) and supplemented with 10% fetal bovine serum and 10% bovine calf serum (Gemini Bio-Products, Woodland, Calif.). Second- or

* Corresponding author. Mailing address: Division of Infectious Diseases, St. John's Cardiovascular Research Center, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Bldg. RB2, 1124 West Carson St., Torrance, CA 90502. Phone: (310) 222-3813. Fax: (310) 782-2016. E-mail: ibrahim@labiomed.org.

FIG. 1. *R. oryzae* spores (A) and germ tubes (B) adhere to endothelial cells, whereas only spores adhere to the subendothelial matrix. *, $P < 0.05$ for endothelial cells versus all other substrates.

third-passage cells were grown to confluency in 96-well or 24-well tissue culture plates (BD Biosciences, Bedford, Mass.) coated with 0.2% gelatin matrix (Vitrogen; Celtrix, Palo Alto, Calif.). All incubations were in 5% CO₂ at 37°C.

Surface labeling with biotin. Spores or germ tubes of *R. oryzae* or *S. cerevisiae* were labeled using the method of Penaver et al. (11). Briefly, organisms (10¹⁰/ml) were suspended in PBS containing 10 mg of *n*-hydroxysuccinimido-biotin (Sigma-Aldrich, St. Louis, Mo.) (dissolved in dimethyl sulfoxide). After 1 h of incubation with shaking at 28°C, the cells were removed and washed four times with 50 mM phosphate buffer (pH 6) and then once with 10 mM phosphate buffer (pH 7.4). Next, biotinylated cells (spores or germ tubes) were incubated for 1 h at room temperature with agitation with extravidin-peroxidase conjugate at a 1:100 dilution in 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl, 0.05% Tween 20, and bovine serum albumin. The extravidin-peroxidase-conjugated biotinylated cells were washed with PBS and resuspended in Hanks balanced salt solution (Irvine Scientific) before use in the adherence assay.

Adherence assay. Adherence of *R. oryzae* or *S. cerevisiae* was carried out in 96-well tissue culture plates coated with HUVECs, fibronectin (10 µg/ml), or 0.2% gelatin in the presence or absence of 10% pooled human serum (Sigma-Aldrich). Adherence to bare plastic was included as a negative control. Various inocula of biotinylated, extravidin-peroxidase-labeled *R. oryzae* spores or germ tubes or *S. cerevisiae* were added in 100-µl aliquots to the 96-well microtiter plate. After a 1-h incubation at 37°C in 5% CO₂, the nonadherent cells were removed by washing with PBS containing 0.05% Tween 20. The substrate mixture containing *o*-phenylenediamine in PBS was then added to the wells per the manufacturer's instructions (Sigma-Aldrich). The plate was incubated in the dark for 20 min, and the color reaction was stopped by adding 25 µl of 3 M sulfuric acid to each well. The color intensity was determined at 490 nm with an automated plate reader (DYNEX Technologies Inc., Chantilly, Va.). Adherence results were expressed as the median optical density at 490 nm of at least quadruplicate wells in three separate experiments.

Endothelial cell injury by viable and nonviable *R. oryzae*. The ability of viable and nonviable *R. oryzae* to damage HUVECs was determined by using the ⁵¹Cr release assay previously described (7). Briefly, HUVECs grown in 24-well tissue

TABLE 1. Effect of serum on *R. oryzae* adherence to HUVECs^a

Sample: inoculum	Median OD (75th quartile, 25th quartile)	
	No serum	+ serum ^b
Spore: 1 × 10 ⁶	0.3 (0.018, 0.017)	0.3 (0.054, 0.004)
Spore: 5 × 10 ⁶	1.2 (0.119, 0.096)	0.8 (0.023, 0.014)*
Spore: 1 × 10 ⁷	1.6 (0.089, 0.049)	1.2 (0.074, 0.078)*
Germ tubes: 1 × 10 ⁶	0.4 (0.025, 0.009)	0.7 (0.021, 0.016)*
Germ tubes: 5 × 10 ⁶	1.3 (0.023, 0.059)	1.1 (0.048, 0.033)
Germ tubes: 1 × 10 ⁷	1.3 (0.031, 0.036)	1.1 (0.057, 0.043)

^a Adherence results were expressed as the median optical density (OD) at 490 nm of at least quadruplicate wells in three separate experiments.

^b *, $P < 0.05$ versus no-serum results by Mann-Whitney U test.

culture plates were incubated with Na₂⁵¹CrO₄ (ICN, Irvine, Calif.) in M-199 medium (2.5 µCi) for 16 h. On the day of the experiment, the unincorporated ⁵¹Cr was aspirated and the wells were washed three times with prewarmed Hanks balanced salt solution. HUVECs were infected with the desired inocula of live or dead *R. oryzae* suspended in 1 ml of RPMI-1640 supplemented with glutamine and 10% pooled human serum at 37°C for selected time intervals. Spontaneous ⁵¹Cr release was determined by incubating HUVECs in RPMI-1640 supplemented with glutamine and 10% pooled human serum without organisms. At the end of the incubation period, 0.5 ml of the medium was aspirated from each well and transferred to glass tubes for determination of ⁵¹Cr activity as a measurement of HUVECs lysis. The wells were then treated with 6 N NaOH for 30 min and rinsed twice with 10% RadiacWash. The NaOH and RadiacWash treatments were combined, and the amount of ⁵¹Cr was measured with a gamma counter. The total amount of ⁵¹Cr incorporated by HUVECs in each well equaled the sum of radioactive counts per minute of the aspirated medium plus the radioactive counts of HUVECs lysed with NaOH and RadiacWash. After the data were corrected for variations in the amount of tracer incorporated in each well, the percentage of specific HUVEC release of ⁵¹Cr was calculated by the following formula: [(experimental release × 2) - (spontaneous release × 2)]/[total incorporation - (spontaneous release × 2)]. Each experimental condition was tested in triplicate with endothelial cells collected from different umbilical cords in three separate experiments.

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

Determination of the relationship between phagocytosis of *R. oryzae* and HUVEC injury. To investigate whether HUVEC injury requires internalization of *R. oryzae*, we studied the effects of disruption of endothelial cell microfilaments with cytochalasin D (Sigma-Aldrich) on damage to HUVECs (3, 7). Selected concentrations of cytochalasin D were added to HUVECs simultaneously with *R. oryzae*, and HUVEC injury was determined as described above. In these experiments, control wells containing cytochalasin D without organisms were included to evaluate the toxicity of cytochalasin D. The highest concentration of cytochalasin D used (200 nM) caused less than 6% specific release of ⁵¹Cr in all these toxicity experiments. Each experimental condition was tested in replicates of three, and each experiment was performed in triplicate with endothelial cells from different umbilical cords.

Statistical analysis. Statistical comparisons were performed by using the nonparametric Steel test for multiple comparisons. Correlations were calculated by the nonparametric Spearman rank sum test. P values of <0.05 were considered significant.

RESULTS

***R. oryzae* specifically adheres to HUVECs in vitro.** To discern if *R. oryzae* could adhere to endothelial cells, we cocultured *R. oryzae* with HUVECs, subendothelial matrix proteins, or plastic. As a negative control, *S. cerevisiae* was also incubated with endothelial cells, and indeed the organism demonstrated no adherence to the endothelial cells (Fig. 1). Because differences in adherence of *R. oryzae* spores and germ tubes to subendothelial matrix proteins had been previously described (1), we tested the adherence of both spores and germ tubes. *R.*

FIG. 2. Interactions of *R. oryzae* spores and germ tubes with endothelial cells. Photomicrographs of endothelial cell monolayers infected with *R. oryzae* spores (A to C) and germ tubes (D to F) after 1 h (A and D), 4 h (B and E), and 8 h (C and F). Original magnification for A, B, D, and E is $\times 20$ and for C and F is $\times 40$. Arrows indicate organisms.

oryzae spores and germ tubes both adhered to endothelial cells in an inoculum-dependent manner (Fig. 1) (inoculum adherence correlation [ρ] = 0.94 for spores and 0.74 for germ tubes; $P < 0.001$ for both by Spearman rank sum test). The adherence of spores to endothelial cells was greater than their adherence to both fibronectin and gelatin for all inocula ($P < 0.05$), although adherence to both fibronectin and gelatin was detectable with the larger inocula. In contrast, germ tubes adhered to endothelial cells for all inocula and demonstrated no adher-

ence to fibronectin, gelatin, or plastic ($P < 0.05$ for endothelial cell adherence versus all other substrates at all inocula).

In separate experiments we tested the ability of pooled human serum to enhance or inhibit *R. oryzae* adherence to HUVECs. Serum slightly inhibited the adherence of larger inocula of *R. oryzae* spores to endothelial cells (Table 1). In contrast, serum almost doubled the adherence of pregerminated *R. oryzae* spores with the small inoculum and had no effect on adherence with the larger inocula.

FIG. 3. Damage to HUVECs is dose and time dependent. (A) Different inocula of *R. oryzae* germ tubes were incubated on endothelial cells for 5 h. *, $P < 0.05$ versus 10^5 inoculum; **, $P < 0.05$ versus 5×10^5 and 5×10^5 inocula. (B) An inoculum of 10^6 *R. oryzae* germ tubes was incubated with endothelial cells for various time intervals. *, $P < 0.05$ versus 2-h incubation.

***R. oryzae* damages endothelial cells in vitro.** To define the ability of *R. oryzae* to damage endothelial cells, we cocultured *R. oryzae* with ^{51}Cr -labeled HUVECs. Morphologically, phase-contrast microscopy revealed that spores inoculated onto HUVECs began to swell and initiate germination by 4 h and by 8 h had formed mature hyphae (Fig. 2A to C). Pregermination of the spores in YPD for 4 to 5 h prior to inoculation onto HUVECs resulted in small germ tubes that were readily quantifiable by counting, as with spores. This is made evident by the small size of the germ tubes even following an hour of incubation on endothelial cells (Fig. 2D). Following 4 h of incubation of pregerminated *R. oryzae* with HUVECs, full hyphal formation was seen, and extensive hyphal mats were seen by 8 h of incubation (Fig. 2E to F). With an inoculum of 5×10^5 , both *R. oryzae* spores and germ tubes damaged HUVECs after a 5-h culture (median damage = 21% for both). Subsequent damage studies were all carried out at 5 h and focused on *R. oryzae* germ tubes because they are likely more reflective of in vivo interactions. The damage to HUVECs mediated by *R. oryzae* germ tubes was inoculum and time dependent (Fig. 3, $p = 0.94$ for inocula and 0.76 for time; $P < 0.0001$ for both). Culture in the presence of serum had no effect on the damage to HUVECs (median ^{51}Cr release = 7% versus 8%, 21% versus 22%, and 35% versus 34% for 1×10^5 , 5×10^5 , and 1

FIG. 4. Multiple *R. oryzae* strains damage endothelial cells more than *Mucor*. Germ tubes (5×10^5) were cultured with endothelial cells for 5 h. *, $P < 0.05$ versus *Mucor* results.

$\times 10^6$ inocula in the absence or presence of serum). Subsequent damage experiments were carried out in the presence of 10% pooled human serum to more closely mimic in vivo conditions and to preserve endothelial cell integrity during prolonged incubation. We also tested the abilities of multiple clinical isolates of *R. oryzae* to damage HUVECs. All *R. oryzae* strains tested germinated equivalently by visual inspection and damaged HUVECs to a similar degree and significantly more than a strain of *Mucor* (Fig. 4).

Damage to endothelial cells from *R. oryzae* requires phagocytosis of the organism but not viability. We have previously found that phagocytosis of *Candida albicans* and *Cryptococcus neoformans* is required for these fungi to damage HUVECs (3, 7). To determine if *R. oryzae*-mediated damage requires a similar interaction with endothelial cells, we tested the ability of *R. oryzae* to damage HUVECs in the presence or absence of membrane inserts. Inserts were added above the HUVECs, and *R. oryzae* was added to the top of the inserts. The presence of the membrane insert completely abolished the ability of *R. oryzae* to damage HUVECs (Fig. 5A), indicating that direct contact of the mycelium is necessary for injury to HUVECs to occur.

We then tested the ability of cytochalasin D, a known inhibitor of HUVEC-mediated phagocytosis (3, 7), to block *R. oryzae*-mediated damage. Increasing concentrations of cytochalasin D increasingly blocked *R. oryzae*-mediated damage (Fig. 5B), demonstrating that HUVEC phagocytosis of the fungus was likely required for the organism to damage HUVECs.

Pilot studies utilizing dead *R. oryzae* as negative controls for damage assays suggested that fungal viability was not required for HUVEC damage. We therefore tested the ability of *R. oryzae* killed by several different techniques to damage HUVECs. Heat-killed, glutaraldehyde-fixed, and ethanol-killed *R. oryzae* mediated damage to HUVECs equivalent to that with viable *R. oryzae* (Fig. 6). To confirm that phagocytosis was required for dead *R. oryzae* to damage HUVECs, we tested the ability of cytochalasin D to abolish damage mediated by dead organisms. Just as for live *R. oryzae*, cytochalasin D significantly blocked damage mediated by heat-killed *R. oryzae* (Fig. 7).

FIG. 5. *R. oryzae*-induced injury to HUVECs requires direct attachment and subsequent internalization by endothelial cells. Germ tubes (5×10^5) were cultured with endothelial cells for 5 h. (A) Damage in the presence or absence of membrane inserts. †, $P < 0.05$ versus results with no membrane inserts. (B) Damage in the presence or absence of cytochalasin D. *, $P < 0.05$ versus results with no cytochalasin D; **, $P < 0.05$ versus results with all other groups.

DISCUSSION

Because angiogenesis is a hallmark of zygomycotic infections, *R. oryzae* interaction with endothelial cells lining blood vessels is likely an integral component of the organism's pathogenic strategy. We find that *R. oryzae* spores and hyphae adhere to and damage HUVECs. This adherence phenomenon is specific, since *R. oryzae* did not adhere to plastic in our assay. In contrast, Bouchara et al. reported that *R. oryzae* spores, but not germ tubes, adhere to plastic (1). The discrepancy between our finding and that of Bouchara et al. (1) is likely due to methodological differences in adherence quantification. Of note, we found that *R. oryzae* spores adhere to subendothelial matrix proteins significantly better than do *R. oryzae* hyphae; however, spores and hyphae adhere equivalently to HUVECs. The disparity of spore and germ tube adherence to subendothelial matrix proteins, but equivalent adherence to HUVECs, indicates that *R. oryzae* adhesins to endothelial cells are likely distinct from the adhesins used to bind to subendothelial matrix proteins.

FIG. 6. Damage to HUVECs from *R. oryzae* is independent of organism viability. *R. oryzae* was killed with heat shock for 1 h at 60°C or incubation in 2.5% glutaraldehyde or 70% ethanol for 30 min. Germ tubes were washed extensively with PBS and then cultured (5×10^5) with endothelial cells for 5 h.

We also found that *R. oryzae* has the ability to damage HUVECs irrespective of the organism's morphology. Endothelial cell damage mediated by *R. oryzae* did not require serum, akin to our previous findings with *C. albicans* (3) but distinct from *Cryptococcus neoformans*, which required serum to cause endothelial cell damage (7).

As with both *C. albicans* and *C. neoformans* (3, 7), *R. oryzae* damage to HUVECs required direct contact to and subsequent phagocytosis of the organism by HUVECs. This is evident by the abolishment of damage in the presence of membrane inserts and cytochalasin D. However, in clear distinction from *C. albicans* and *C. neoformans* (3, 7), *R. oryzae* does not need to be viable to damage HUVECs. This damage caused by *R. oryzae* is also distinct from that from *Aspergillus fumigatus* in that nonviable hyphae, but not spores, of *A. fumigatus* have been reported to cause endothelial cell damage (10). By contrast, in our study both nonviable spores and hyphae of *R. oryzae* caused damage to HUVECs, possibly indicative of the presence of a toxin in *R. oryzae*.

Adherence to, internalization by, and subsequent injury to HUVECs by *R. oryzae* likely occur in vivo during mucormycosis. These processes may contribute to the ischemic necrosis often seen with mucormycosis infections. The capability of

FIG. 7. Damage mediated by dead *R. oryzae* also requires phagocytosis by HUVECs. Germ tubes (5×10^5) were cultured with endothelial cells for 5 h in the presence of 200 nM cytochalasin D. *, $P < 0.05$ versus results with no cytochalasin D.

dead *R. oryzae* to cause damage to human cells indicates that administration of fungicidal antibiotics that result in *R. oryzae* death may fail to prevent surrounding tissue necrosis in vivo. This may be one reason why *R. oryzae* infections are so refractory in patients.

In conclusion, we show that *R. oryzae* interacts with endothelial cells, likely via specific receptors that induce its own phagocytosis. *R. oryzae* damage to endothelial cells appears to require phagocytosis but does not require organism viability. These results elucidate the crucial interaction between *R. oryzae* and endothelial cells and may have implications for treatment of this refractory pathogen.

ACKNOWLEDGMENTS

We thank the PCRC nurses at Harbor-UCLA Medical Center for collecting umbilical cords and Toyota USA for donating the Olympus phase-contrast microscope.

This study was supported by a New Investigator Award in Molecular Pathogenic Mycology from the Burroughs Wellcome Fund to A.S.I. A.S.I. is also supported by an RO3 AI054531 grant from the National Institute of Allergy and Infectious Diseases. B.J.S. is supported by a Public Health Service grant, KO8 AI060641-01. J.E.E. is supported by an unrestricted Freedom to Discover Grant for Infectious Disease from Bristol Myers Squibb. Research described in this report was conducted at the research facilities of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center.

REFERENCES

1. Bouchara, J. P., N. A. Oumeziane, J. C. Lissitzky, G. Larcher, G. Tronchin, and D. Chabasse. 1996. Attachment of spores of the human pathogenic fungus *Rhizopus oryzae* to extracellular matrix components. *Eur. J. Cell Biol.* **70**:76–83.
2. Edwards, J., Jr. 1989. Zygomycosis, p. 1192–1199. In P. Hoeprich and M. Jordan (ed.), *Infectious disease*, 4th ed. J.B. Lippincott Co., Philadelphia, Pa.
3. Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Lockett. 1995. Penetration and damage of endothelial cells by *Candida albicans*. *Infect. Immun.* **63**:976–983.
4. Fu, Y., H. Lee, M. Collins, H. F. Tsai, B. Spellberg, J. E. Edwards, Jr., K. J. Kwon-Chung, and A. S. Ibrahim. 2004. Cloning and functional characterization of the *Rhizopus oryzae* high affinity iron permease (rFTR1) gene. *FEMS Microbiol. Lett.* **235**:169–176.
5. Hussain, S., N. Salahuddin, I. Ahmad, I. Salahuddin, and R. Joorna. 1995. Rhinocerebral invasive mycosis: occurrence in immunocompetent individuals. *Eur. J. Radiol.* **20**:151–155.
6. Ibrahim, A. S., J. E. J. Edwards, and S. G. Filler. 2003. Zygomycosis, p. 241–251. In W. E. Dismukes, P. G. Pappas, and J. D. Sobel (ed.), *Clinical mycology*. Oxford University Press, New York, N.Y.
7. Ibrahim, A. S., S. G. Filler, M. S. Alcouloumre, T. R. Kozel, J. E. Edwards, Jr., and M. A. Ghannoum. 1995. Adherence to and damage of endothelial cells by *Cryptococcus neoformans* in vitro: role of the capsule. *Infect. Immun.* **63**:4368–4374.
8. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Investig.* **52**:2745–2756.
9. Kwon-Chung, K. J., and J. E. Bennett. 1992. Mucormycosis, p. 524–559. In *Medical mycology*. Lea & Febiger, Philadelphia, Pa.
10. Lopes Bezerra, L. M., and S. G. Filler. 2004. Interactions of *Aspergillus fumigatus* with endothelial cells: internalization, injury, and stimulation of tissue factor activity. *Blood* **103**:2143–2149.
11. Penalver, M. C., J. E. O'Connor, J. P. Martinez, and M. L. Gil. 1996. Binding of human fibronectin to *Aspergillus fumigatus* conidia. *Infect. Immun.* **64**:1146–1153.
12. Ribes, J. A., C. L. Vanover-Sams, and D. J. Baker. 2000. Zygomycetes in human disease. *Clin. Microbiol. Rev.* **13**:236–301.
13. Sugar, A. M. 1995. Agent of mucormycosis and related species, p. 2311–2321. In G. Mandell, J. Bennett, and R. Dolin (ed.), *Principles and practices of infectious diseases*, 4th ed. Churchill Livingstone, New York, N.Y.

Editor: T. R. Kozel