Effect of Endothelial Cells on Phagocyte-Mediated Anticryptococcal Activity

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The anticryptococcal activity of peripheral blood polymorphonuclear leukocytes (PMN) and monocytes was compared on plastic versus human umbilical vein endothelial cell surfaces. Various amounts of PMN and monocytes were incubated on plastic or endothelial surfaces and then challenged for 18 h with Cryptococcus neoformans. Both phagocyte populations exhibited significantly more anticryptococcal activity on an endothelial cell monolayer than on plastic. Prestimulating the endothelial cell monolayer with interleukin-1 augmented the antifungal activity of PMN but not that of monocytes. In the absence of phagocytes, endothelial cells lacked activity. Blocking antibodies directed against endothelial adhesion molecules ICAM-1 and ELAM-1 did not affect PMN-mediated inhibition of fungal growth. Recombinant interleukin-1 and interleukin-8 (two cytokines secreted by endothelial cells) activated neutrophils for modestly enhanced antifungal activity. However, supernatants derived from endothelial cells, as well as neutralizing antibodies directed against the endothelial cell-derived cytokines interleukin-8 and granulocyte-macrophage colony-stimulating factor failed to augment PMN antifungal activity. PMN viability after 18 h was diminished on plastic compared with endothelial surfaces. While the percentages of C. neoformans bound to neutrophils were similar on both surfaces, the patterns of binding were markedly different: on endothelial (but not plastic) surfaces, most cryptococci were surrounded by greater than five PMN. Thus, phagocyte-mediated inhibition of cryptococcal growth is enhanced on endothelial monolayers compared with plastic surfaces, possibly as a result of differences in phagocyte viability and patterns of binding. Bolstering the activity of circulating phagocytes by stimulating endothelial cells may be of relevance in the treatment of patients with or at risk for cryptococcosis.

Cryptococcal infections are a significant cause of morbidity and mortality in individuals with compromised cell-mediated immunity, especially those with hematologic malignancies, steroid use, and human immunodeficiency virus infection. Following respiratory acquisition of the organism, the pulmonary system provides the first line of defense against cryptococcal disease (5, 19, 36). In experimental murine pulmonary infections, early clearance of Cryptococcus neoformans is rapidly accomplished by polymorphonuclear leukocytes (PMN), while mononuclear cells predominate later in the inflammatory infiltrate (11). If the organism overwhelms the lungs' defenses, hematogenous dissemination to the meninges and other organ systems may occur. Indeed, patients with cryptococcosis frequently have cryptocococemia, which, when found, is a poor prognostic sign (7, 29). Thus, peripheral blood phagocytes presumably form an important defense against disseminated cryptococcosis.

Other investigators have demonstrated that PMN and monocytes have fungicidal activity against C. neoformans in in vitro assays of 4 h or less (8, 28). However, in those assays, a significant percentage of the organisms was not killed and the fate of the surviving yeast cells was not determined. Moreover, phagocytes and fungi were co-incubated in plastic reaction vessels. Recent studies have emphasized the importance of the culture surface to phagocyte function. For example, monocyte-derived macrophages cultured on fibronectin surfaces completely inhibited the growth of C. neoformans opsonized with pooled human serum (PHS), while those cultured on plastic did not (20).

The physiological function of blood PMN is critically dependent upon their ability to interact with endothelial cells. Recognition of PMN by endothelial cells is promoted by over 10 adhesion receptors (8, 24, 30–32). In the presence of inflammation, many of the adhesion receptors are upregulated so as to facilitate intercellular binding and subsequent extravasation of PMN across the endothelium (9, 26, 30, 32). For example, endothelial-leukocyte adhesion molecule 1 (ELAM-1) and intercellular adhesion molecule 1 (ICAM-1) are maximally expressed on human endothelial cells 4 and 24 h, respectively, following exposure to the proinflammatory cytokine interleukin-1 (IL-1) (1, 4, 9, 32). The purpose of this study was to evaluate whether the activity of human blood phagocytes against C. neoformans is modified in the presence of unstimulated and cytokine-stimulated endothelial cell surfaces.

MATERIALS AND METHODS

Materials. Reagents used were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Medium used, except where stated otherwise, was RPMI 1640 (Bio-Whittaker, Walkersville, Md.) supplemented with penicillin, streptomycin, and l-glutamine. For growth inhibition assays, flat-bottom 96-well "half-area" polystyrene tissue culture plates (number 3696; Costar, Cambridge, Mass.) were used. Flat-bottom full-area 96-well polystyrene tissue culture plates (number 25860; Corning Glass Works, Corning, N.Y.) were used for binding and PMN viability assays. PHS was obtained by combining sera from at least 10 healthy donors, stored in aliquots at −70°C, and used at a final concentration of 10%. Monoclonal antibodies (MAb) directed against CD18 (TSI/18), CD11a (TSI/22), IL-8 (neutralizing MAb, 5-12-14), ELAM-1 (blocking MAb, H18/7),
ELAM-1 (nonblocking MAb, H4/18), and ICAM-1 (Hu5/3) were generously provided by F. W. Luscinuskas (Brigham and Women’s Hospital, Boston, Mass.). Neutralizing MAb specific for granulocyte-macrophage colony-stimulating factor (GM-CSF) (HGM2/4.8 and HGM2/3.1) were obtained from Genetics Institute (Cambridge, Mass.). Cytokines used were IL-1β (Genzyme, Cambridge, Mass.) and IL-8 (Cellular Products, Buffalo, N.Y.). N^2-monomethyl-L-arginine, monoacetate (NMMA) was obtained from Calbiochem (La Jolla, Calif.).

**C. neoformans.** The encapsulated, serotype A strain 145 of *C. neoformans* used for all studies was a gift from Thomas Mitchell. This cryptococcal strain has been shown to be susceptible to killing by human PMN and monocytes (28). The organism was maintained on asparagine minimal agar medium at 30°C, as in previous studies (18, 20–22). Under these conditions, the diameter of the organisms and width of the capsule were 4.35 ± 0.20 μm and 0.48 ± 0.19 μm (mean ± standard deviation), respectively.

**HUVEC.** Human umbilical vein endothelial cells (HUVEC; Cell Systems, Kirkland, Wash.) were harvested by using collagenase from a pool of 30 umbilical cords and were used at cell passages 2 and 3. The monolayers were maintained in culture with medium consisting of M199 (GIBCO, Grand Island, N.Y.), 20% heat-inactivated fetal calf serum, endothelial growth factor (15 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), thymidine (1 μM), heparin (10 μg/ml), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (10 mM), and glutamine (2 mM) in tissue culture flasks (25 cm², number 25106; Corning Glass Works), coated with 5% gelatin (Difco Laboratories, Detroit, Mich.). HUVEC were grown to confluence in tissue culture wells precoated with 5% gelatin. Purity was verified by indirect immunofluorescence labeling with polyclonal rabbit immunoglobulin G directed against human factor VIII.

**IL-1 stimulation of HUVEC.** Cultured HUVEC monolayers were incubated with IL-1 (20 U/ml) for 3 or 24 h at 37°C. Unless stated otherwise, IL-1-treated HUVEC refers to HUVEC treated for 4 h. Treated monolayers were then washed three times with medium to remove residual IL-1.

**Isolation of PMN and monocytes.** Human peripheral blood was obtained by venipuncture from normal volunteers and anticoagulated with 10 U of heparin per ml of blood. PMN were separated by centrifugation (550 × g) on a Ficoll-Hypaque density gradient, subjected to saline lysis, and resuspended in medium at the desired concentration (27). Monocytes were obtained by the method of Freundlich et al. (10). Briefly, heparinized whole blood was centrifuged at 1,000 × g at 4°C for 15 min. The plasma supernatant was used to coat a plastic petri dish previously treated with 2% gelatin. The buffy coat was diluted 1:3 with phosphate-buffered saline (PBS; GIBCO) and centrifuged on a Ficoll-Hypaque density gradient. The resultant monolayer was washed, resuspended in RPMI 1640 supplemented with 20% fetal calf serum, and incubated for 1 h at 37°C on the gelatin-plasma-coated petri dish. Nonadherent cells were aspirated. Adherent monocytes were loosened with 0.02% EDTA, recovered by gentle scraping with a rubber policeman, washed, and resuspended in RPMI 1640 at the desired concentration. Monocytes obtained by this method were 73% ± 4% (mean ± standard error of the mean [SEM] of three experiments) pure as determined by nonspecific esterase staining (Sigma Diagnostics, kit 91-A). The remaining cells had the characteristics of lymphocytes as determined by flow cytometry and hematoxylin staining.

**Growth inhibition assay.** PMN or monocytes were added to uncoated polystyrene wells (hereafter referred to as plastic surfaces) and to wells containing untreated and IL-1-treated HUVEC monolayers. Each sample was prepared in triplicate. In some experiments, saturating concentrations of MAb specific for GM-CSF (50 μg/ml), IL-8 (20 μg/ml), ICAM-1 (25 μg/ml), ELAM-1 (blocking, 20 μg/ml), ELAM-1 (nonblocking, 20 μg/ml), CD11a (1:200 ascites dilution), CD18 (1:200 ascites dilution), or NMMA (100 μM) were added to the IL-1-stimulated endothelial monolayer for 20 to 30 min prior to the addition of PMN and were allowed to remain throughout the subsequent incubation. After 1 h, the well contents were challenged with 10⁷ CFU of *C. neoformans* in the presence of 10% PHS. Following an 18-h incubation at 37°C (and at 4°C for the control specimens) 0.1% Triton X-100 was added to the wells, and the contents of the wells were transferred to test tubes containing 1 ml of distilled H₂O and sonicated under conditions shown to lyse the leukocytes without affecting fungal viability. Microscopic observations ensured that the entire contents of the wells were transferred, cell lysis was complete, and clumping of organisms did not occur. Dilutions and spread plates were then performed, and CFU were counted after 72 h. Percent growth was then calculated as follows: [(experimental CFU)/(mean CFU at 4°C) – 1] × 100. Thus, a value of zero indicates that the number of CFU at the start and conclusion of the incubation were the same. Values of 100, 200, and 400% indicate that the fungi underwent an average of one, two, and three replications, respectively. Negative values indicate that the number of CFU decreased during the incubation, and therefore fungal killing had taken place (17, 20, 21). However, with this assay, the exact extent of killing or fungistasis cannot be quantitated since, during the 18-h incubation period, some fungi may grow while others are killed.

**Detection of nitric oxide.** Production of nitrite, a stable end-product of nitric oxide generation, was determined in supernatants from endothelial cells incubated for up to 72 h by a colorimetric assay (15). Briefly, equal amounts of supernatant and Griess reagent were incubated for 10 min, and ΔA_m was determined.

**PMN viability assay.** PMN were suspended at 10⁷/ml in PBS containing 0.2% bovine serum albumin and 100 μCi of sodium chromate (³¹Cr; New England Nuclear, Boston, Mass.) per ml for 1 h with rocking at 37°C (6). Following three washes, 1 × 10⁵ or 3.3 × 10⁵ PMN were added to experimental wells in a total volume of 200 μl and incubated for 18 h at 37°C. Supernatants were collected and counted on a gamma counter. Percent ³¹Cr release from cells lysed with 1 M NaOH, whereas background counts per minute represents medium alone.

**Binding assay.** Because of the difficulties inherent in the use of light microscopy to accurately identify *C. neoformans* and PMN in the presence of an endothelial monolayer, a fluorescence binding assay was developed. Live *C. neoformans* was labeled by rotation with fluorescein isothiocyanate (FITC; 1 mg/ml) at 37°C for 30 min. The labeled yeast cells were then washed five times with PBS and resuspended at 5 × 10⁶ cells per ml. PMN were labeled with the lipophilic carbocyanine fluorescent dye 1,1'-dioctadecyl-3,3',3''-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, Oreg.) by a modification of the method of Lo et al. (23). Cells at 10⁷/ml were rotated in PBS containing
cryptococcal inhibition when $3 \times 10^9$ PMN were added per well. If PHS was either heated to 56°C or omitted entirely from the system, PMN-mediated antifungal activity was completely abolished (data not shown). When these experiments were conducted with a 2-h rather than an 18-h incubation, no difference in the number of CFU was observed on an endothelial versus a plastic surface (data not shown).

**Effect of MAb specific for endothelial and PMN surface molecules on cryptococcal growth.** Having established that fungal growth was inhibited following incubation of PMN on IL-1-stimulated and untreated endothelial monolayers, we next examined the contribution of selected endothelial and leukocyte adhesion molecules to the diminished cryptococcal growth (Table 1). Addition of blocking MAb directed against the endothelial cell adhesion molecules ELAM-1 and ICAM-1 had no effect on fungal growth at both PMN concentrations tested. Paradoxically, there was less fungal growth in wells containing MAb against the leukocyte integrin LFA-1 (CD11a) in the presence of $1 \times 10^5$ (but not $3 \times 10^5$) PMN. Conversely, wells containing MAb against CD18 and $3 \times 10^5$ (but not $1 \times 10^5$) PMN had greater fungal growth. Since CD18 forms part of two complement receptors (CR3 and CR4), it remains possible that the increased growth seen in the presence of anti-CD18 MAB was secondary to decreased PMN recognition of their fungal targets. However, there was no effect on fungal growth when anti-CD18 MAB was added to wells containing PMN but no HUVEC (data not shown).

**Effect of soluble endothelial products on cryptococcal growth.** The next set of experiments sought to assess whether neutrophil-activating cytokines released from HUVEC could have contributed to the enhanced anticyptococcal activity of PMN on an endothelial monolayer. The effects of recombinant cytokines, supernatants from stimulated HUVEC, and neutralizing anticytokine MAB were evaluated. Compared with untreated PMN, there was a statistically significant reduction in cryptococcal growth in the presence of PMN treated with 0.2 and 20 U of IL-1 per ml (Table 2), or 10 ng of IL-8 per ml (Table 3). Supernatants obtained from unstimulated and IL-1-treated HUVEC monolayers did not modify cryptococcal growth when incubated with $10^5$ PMN on plastic (data not shown). Addition of neutralizing MAB specific for IL-8 or GM-CSF did not affect
the inhibition of cryptococcal growth mediated by PMN on an IL-1-stimulated HUVEC monolayer (Table 4).

The anticryptococcal activity of PMN on stimulated HUVEC was not affected by the addition of NMMA (Table 4). NMMA inhibits synthesis of nitric oxide (15), a reactive nitrogen intermediate with known anticryptococcal activity (12). Moreover, nitric oxide was not detected in supernatants from IL-1-stimulated and unstimulated HUVEC (data not shown).

Quantitation of $^{51}$Cr release as a measure of PMN viability. The viability assay measured PMN survival on plastic and endothelial surfaces under the same conditions used in the growth inhibition assay (Fig. 2). In five separate experiments using two PMN concentrations, $^{51}$Cr release was significantly greater for PMN incubated on plastic than for those incubated on untreated and IL-1-stimulated HUVEC monolayers. Although these results were significant, there was considerable experiment-to-experiment variability. Thus, in three of the five experiments, only small differences were noted in $^{51}$Cr release between the groups, whereas in the other two experiments $^{51}$Cr release differed by as much as 36% (data not shown).

Binding assay. We next measured the ability of PMN to bind C. neoformans on plastic, HUVEC, and IL-1-treated HUVEC surfaces (Fig. 3 and 4). When PMN were incubated with C. neoformans in the presence of PHS, over 80% of the yeast cells were bound by the phagocytes, regardless of whether HUVEC were present. However, the pattern of binding seen on plastic surfaces differed significantly from that seen on HUVEC. On the plastic surface, the majority of bound C. neoformans cells were associated with only one to five PMN, whereas on the unstimulated or IL-1-stimulated HUVEC surface, over 65% of the yeast cells were surrounded by greater than five PMN. The same pattern of binding was seen even if the HUVEC monolayer was fixed with methanol prior to the binding assay (data not shown). In the absence of PHS, significant binding was not seen (data not shown).

Effect of endothelial cells on monocyte antifungal activity. The final set of experiments examined whether endothelial cells enhanced the anticryptococcal activity of monocytes

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**TABLE 3. IL-8 stimulation of PMN**

<table>
<thead>
<tr>
<th>IL-8 (ng/ml)</th>
<th>% Growth$^a$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>424 ± 54</td>
<td>0.22</td>
</tr>
<tr>
<td>0.1</td>
<td>478 ± 59</td>
<td>0.04</td>
</tr>
<tr>
<td>1</td>
<td>363 ± 39</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^a$ PMN were incubated with IL-8 on plastic for 1 h and then challenged for 18 h with C. neoformans.

$^b$ Data represent means ± SEM of four triplicate experiments.

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**TABLE 4. Effects of MAb against soluble endothelial products on cryptococcal growth**

<table>
<thead>
<tr>
<th>MAb/inhibitor</th>
<th>Specificity</th>
<th>$1 \times 10^5$ PMN</th>
<th>$3.3 \times 10^5$ PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MAb/inhibitor</td>
<td>ND</td>
<td>350 ± 53</td>
<td>350 ± 53</td>
</tr>
<tr>
<td>HGM2/4.8</td>
<td>GM-CSF</td>
<td>260 ± 38</td>
<td>260 ± 38</td>
</tr>
<tr>
<td>5-12-14</td>
<td>IL-8</td>
<td>367 ± 44</td>
<td>367 ± 44</td>
</tr>
<tr>
<td>Mixture$^a$</td>
<td>GM-CSF/IL-8</td>
<td>321 ± 44</td>
<td>321 ± 44</td>
</tr>
<tr>
<td>NMMA</td>
<td>Nitric oxide</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ PMN were incubated with MAb or inhibitor on IL-1-treated HUVEC monolayers for 1 h and then challenged for 18 h with C. neoformans.

$^b$ Data represent means ± SEM of five and two triplicate experiments for $1 \times 10^5$ and $3.3 \times 10^5$ PMN, respectively.

$^c$ Mixture comprises MAb directed against GM-CSF and IL-8.

$^d$ ND, not determined.

(Fig. 5). No significant difference in growth was apparent when $10^5$ monocytes were incubated on HUVEC compared with plastic. However, when the number of monocytes was increased to $3.3 \times 10^5$/well and $1 \times 10^5$/well, cryptococcal growth was significantly less on the HUVEC monolayer than on plastic. Results were similar regardless of whether the HUVEC monolayer was stimulated with IL-1 (data not shown). As with the PMN studies, when these experiments were conducted with a 2-h rather than an 18-h incubation, no difference in the number of CFU was observed on endothelial versus plastic surfaces (data not shown).

**DISCUSSION**

The data presented here demonstrate that the anticryptococcal activity of peripheral blood phagocytes is significantly enhanced when cultured on an endothelial monolayer compared with a plastic surface. The inhibition of cryptococcal growth in the presence of PMN (but not monocytes) is further enhanced when the endothelial monolayer is stimulated with IL-1. In the absence of peripheral blood phagocytes, HUVEC, whether stimulated with IL-1 or in the basal state, are devoid of anticryptococcal activity.

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**FIG. 2. Effects of endothelial cells on PMN viability.** $^{51}$Cr release was calculated following an 18-h incubation of $^{51}$Cr-labeled PMN on plastic, HUVEC, and IL-1-stimulated HUVEC surfaces. For both concentrations of PMN, P ≥ 0.01 comparing $^{51}$Cr release on plastic with endothelial surfaces. Data represent means ± SEM of five triplicate experiments.

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IL-1 stimulation of a HUVEC monolayer enhances the adhesiveness of the monolayer for both PMN and monocytes (2, 3). We evaluated endothelial adhesion molecules ELAM-1 and ICAM-1 and leukocyte integrins CD11a and CD18 as possible contributors to the observed antifungal activity. We postulated that IL-1-promoted PMN adhesion to endothelial cell surface molecules would activate the PMN, resulting in enhanced anticytotoxic activity. However, blocking antibodies directed against ELAM-1, ICAM-1, or CD11a did not inhibit antifungal activity, whereas CD18 MAAb had an effect only at the higher PMN concentration evaluated. It should be noted, though, that cell surface molecules not tested, such as the recently described ICAM-R (34) or other as-yet-undiscovered adhesion molecules, may contribute to the enhanced activity.

During inflammation, endothelial cells are activated by various stimuli to secrete the neutrophil-activating cytokines IL-1, IL-8, and GM-CSF (25, 31). To examine the potential contribution of these cytokines to the enhanced anticytotoxic activity manifested by PMN in the presence of activated HUVEC, we tested the effects of recombinant cytokines, neutralizing anticytokine antibodies, and supernatants obtained from stimulated HUVEC. Recombinant IL-1, at concentrations of 0.2 and 20 U/ml, activated neutrophils for enhanced antifungal activity, although the effects were modest (Table 2). Similarly, IL-8, at the highest concentration tested, significantly activated PMN for enhanced activity (Table 3). The inability of supernatants obtained from stimulated HUVEC to augment PMN antifungal activity and the lack of effect of neutralizing antibodies directed against IL-8 and GM-CSF (Table 4) suggest a major role for endothelial cell-derived cytokines in our system. However, a role for cytokines is not precluded, as it remains possible that endothelial cell-derived cytokines are produced locally in high concentrations or at areas of tight contact between HUVEC and PMN. In any event, our data demonstrating the capacity of IL-1 and IL-8 to enhance the anticytotoxic activity of PMN underscore the potential role of these cytokines in modulating host defenses against cryptococcosis. IL-1 and/or IL-8 derived from endothelial cells, macrophages, or other cell types may activate PMN in inflammatory foci to contain the fungal burden.

The reactive nitrogen intermediate nitric oxide has been shown to contribute to the ability of activated murine macrophages to inhibit and kill C. neoformans (12, 18). Recently, the machinery for generating nitric oxide in human endothelial cells has been demonstrated (13). However, two lines of evidence suggest that generation of nitric oxide was not critical for the enhanced antifungal effects of PMN seen when HUVEC were present. First, addition of NMMA, a competitive inhibitor of nitric oxide synthesis, had no effect in our system. Second, nitrite (a sensitive marker for nitric oxide generation) was not present in detectable quantities in the supernatants of IL-1-treated HUVEC.

The finding that PMN viability, as measured by 51Cr release, was significantly less on plastic than on HUVEC (Fig. 2) raises the possibility that the diminished antifungal activity of PMN cultured on plastic was due, at least in part, to greater death of the PMN over the course of the 18-h assay. Other investigators have shown that culture conditions can greatly influence the long-term viability and antimicrobial activity of PMN (16). Conversely, PMN death has been postulated to be beneficial to host defenses by causing release of calprotectin, a PMN cytosolic protein with antimicrobial properties (33).

Information from the binding assay may provide insight into the mechanism(s) behind the greater inhibition of cryp-
toccocal growth on a HUVEC monolayer. Although the percentages of C. neoformans bound by PMN were approximately the same in the presence and absence of HUVEC, the patterns of binding were markedly different. On HUVEC (but not plastic) surfaces, most cryptococci were surrounded by large clusters composed of greater than five PMN (Fig. 3 and 4). This effect appeared to be secondary to the surface properties of the HUVEC, since fixing the monolayers with methanol did not change the distribution of yeast cells and PMN. Having multiple phagocytes converge upon individual yeast cells could be beneficial to host defenses. First, those yeast cells which are bound to the surface of the phagocyte without being internalized could be attacked by multiple cells (14, 22). Second, intracellular yeasts which grow and destroy the PMN could still be susceptible to attack by the surrounding PMN.

The finding that PMN- and monocyte-mediated inhibition of cryptococcal growth is enhanced on an endothelial monolayer underscores the importance of conducting in vitro experiments using conditions that attempt to mimic the situation found in vivo. These results may be of relevance to patients with AIDS or other severe defects of cell-mediated immunity. Such patients must rely heavily on phagocytic defenses against cryptococcosis. Bolstering the activity of circulating PMN by stimulating endothelial cells could serve as an adjuvant to the agents available to combat disseminated cryptococcosis.

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