Immortalized Human Brain Endothelial Cell Line HCMEC/D3 as a Model of the Blood-Brain Barrier Facilitates In Vitro Studies of Central Nervous System Infection by *Cryptococcus neoformans*[▽]

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Cryptococcus neoformans cells must cross the blood-brain barrier prior to invading the central nervous system. Here we demonstrate that the immortalized human brain endothelial cell line HCMEC/D3 is a useful alternative to primary brain endothelial cells as a model of the blood-brain barrier for studies of central nervous system infection.

Cryptococcus neoformans is a fungal human pathogen that causes meningitis in a predominantly immunocompromised population (26). To invade the central nervous system (CNS), cryptococcal cells must cross the blood-brain barrier (BBB) (2). Despite efforts to understand the propensity of this pathogen for the CNS, little progress has been made in the past couple of decades (9, 18–20). The major reasons for this have been the inability to recapitulate the properties of the BBB in vitro and the many challenges posed by BBB studies using live animals (13, 21, 22, 24, 32, 35). Although commercially generated primary human brain microvascular endothelial cells (HBMEC) are now available for BBB studies, there are several disadvantages to developing models of the BBB using these cells. Mainly these primary cells are unstable after a limited number of passages, and they can be very expensive. The alternative, obtaining primary HBMEC from discarded brain tissues, is also undesirable since the process is labor-intensive and introduces variability from batch to batch (4, 8). To facilitate the study of the BBB in vitro, researchers have tried to develop human brain endothelial cell lines that retain critical features of primary cells, such as the expression of endothelial cell markers, transporters, and tight junctional proteins (1, 15, 23, 25, 27, 29, 30, 33, 34, 36). The recent development of one particular line of immortalized human brain endothelial cells (HCMEC/D3) that recapitulates many of the key characteristics of primary brain endothelial cells without the need to coculture with glial cells is proving to be a promising cell line for in vitro studies of the BBB (36). Indeed, the HCMEC/D3 cell line has already been successfully used as a BBB model in several studies, further attesting to its high quality and its potential to replace primary cells for in vitro BBB studies (10, 11, 12, 14–16, 28, 31, 37).

Here we show that the HCMEC/D3 cell line can serve as a useful in vitro model of the BBB to study the mechanisms used by *C. neoformans* to breach the brain endothelium and enter the CNS. In order to test the feasibility of this cell line as a BBB model to study the migration of *C. neoformans* across the BBB, a transcytosis assay was used. This assay consisted of a transwell apparatus with endothelial cells growing in rich endothelial growth medium (EGM-2; Lonza) on a collagen-coated porous membrane (8 μm; Bioscience) (Fig. 1A) (4, 8). The HCMEC/D3 cells used here were between passages 25 and 35. HCMEC/D3 cells were seeded based on the growth area ratio. A confluent monolayer in a culture flask of 25 cm^2 was trypsinized and resuspended in 12 ml of medium. The ratio 12 ml/25 cm^2 (0.5 ml/1 cm^2) was used to determine how much volume was needed to seed the insert. Seeding 500 μl was essentially the equivalent of adding 1 cm^2 of confluent monolayer to the transwell apparatus. A 50% seeding would mean using 250 μl of trypsinized suspension from a fully confluent monolayer to seed the transwell apparatus. Once added to the transwell apparatus, the HCMEC/D3 cells were cultivated for approximately 6 to 7 days at 37°C and 5% CO_2. Prior to starting transcytosis, the medium of both transwell chambers was changed to include 2.5% human serum. The medium was changed 1 day after seeding from 1× strength to 0.5× strength 24 h before the assay. The use of the lower-strength medium was required to reduce the growth factors in the medium as the cells reached confluence so that the monolayer could differentiate, rather than allowing individual cells to continually divide, since cell division appeared to impair the permeability of the HCMEC/D3 barrier (36). Since cell growth in 0.25×-strength medium is very slow, this medium was not used. Also, the multiplication rate of fungal cells in the lower chamber was not significant when reduced-strength medium (0.5×) was used and when CFU counts were taken prior to 24 h.

Cells of a wild-type strain of *C. neoformans* (H99; 1×10^5 cells per well) were added to the top chamber, and the number of cryptococcal cells that crossed the endothelial cell barrier was monitored over time by taking aliquots (100 μl) from the

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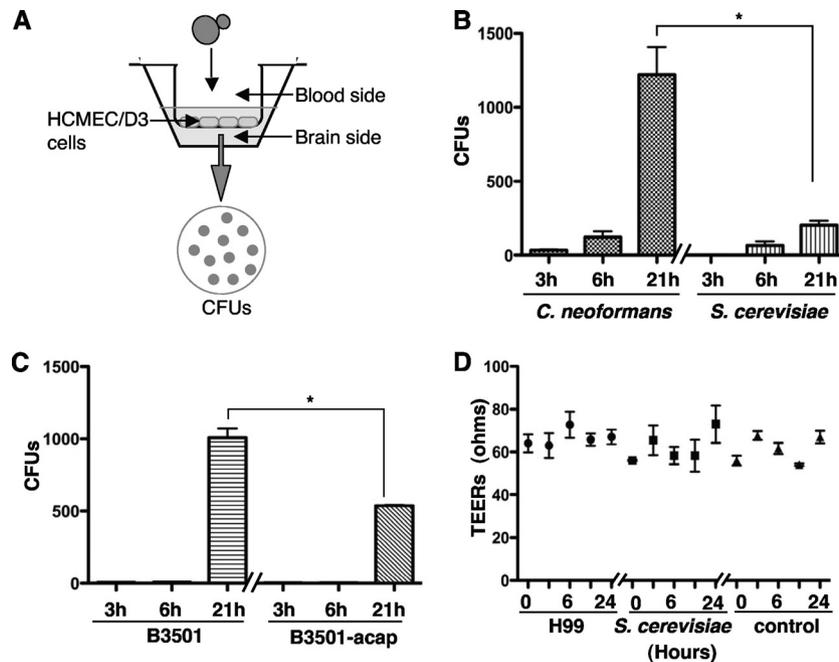
FIG. 1. *C. neoformans* crosses the HCMEC/D3 cell line, an in vitro model of the BBB. (A) The in vitro BBB model consists of a transwell apparatus separating the luminal (blood) and abluminal (brain) sides of the BBB. HCMEC/D3 cells were grown in the top transwell chamber on a collagen-coated microporous membrane (8 μm). Fungal cells (1×10^5 cells) were added to the top chamber and collected subsequently from the bottom chamber for CFU determination. (B) A wild-type strain of *C. neoformans* (H99) readily crossed from the luminal to the abluminal side of the in vitro BBB model. *S. cerevisiae* was used as a control strain to test the ability of the in vitro barrier to exclude a nonpathogenic yeast. ∗, results determined by *t* test ($P < 0.05$). Data are expressed as means ± standard deviations. (C) The contribution of the capsule of *C. neoformans* cells to BBB crossing was examined using a wild-type encapsulated strain B3501 and its isogenic acapsular counterpart. The encapsulated strain crossed the endothelial cell monolayer significantly better than the acapsular strain. ∗, results determined by *t* test ($P < 0.05$). Data are expressed as means ± standard deviations. (D) TEER for the in vitro BBB model was monitored over time in the absence (control) or presence of cryptococcal cells or yeast cells. TEER values were not significantly different before and after the addition of fungal cells, indicating that the barrier function of the HCMEC monolayer was not compromised by *C. neoformans*. Data are expressed as means ± standard deviations. All *Cryptococcus neoformans* strains (H99 *MAT*α serotype A, B3501, and acapsular B3501 [B3501-acap]) and a *Saccharomyces cerevisiae* strain (W303) were recovered from 15% glycerol stocks stored at −80°C prior to use. Strains were maintained on YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium. Fungal cells were cultured in YPD medium at 30°C for 24 h. The HCMEC/D3 cell line was obtained under a license from INSERM, France. The original brain endothelial cells used for generating HCMEC/D3 were isolated from the temporal lobe of an adult female with epilepsy (36).

bottom chamber for CFU determination. Our results indicated that cryptococcal cells crossed the HCMEC/D3 monolayer significantly better than the negative control, *Saccharomyces cerevisiae*, by 21 h (Fig. 1B). This is consistent with the unique ability of *C. neoformans* to invade the CNS. We also found that less than 8% of fungal cells added to the top chamber crossed the monolayer. This is less than previously reported, suggesting that the HCMEC/D3 cells may have formed a more robust, restrictive barrier than primary cultures of brain endothelium (4). Unlike *S. cerevisiae* cells, *C. neoformans* cells each maintain a large polysaccharide capsule that serves as a major virulence factor. In order to examine whether the capsule contributed to the migration of *C. neoformans* across the in vitro barrier, an isogenic capsular and acapsular strain pair were used (Fig. 1C). We found that the wild-type encapsulated B3501 strain crossed significantly better than its isogenic acapsular counterpart (Fig. 1C), consistent with previous results demonstrating that strains with a larger capsule tended to cross the in vitro barrier better than strains with a smaller capsule (4, 5, 8).

The capsule may be of particular importance because hyaluronic acid, present mainly in the innermost layer of the capsule, has been shown to associate with a CD44 glycoprotein receptor on the surface of the brain endothelium (3, 17, 19).

The interaction between CD44 and hyaluronic acid appears to be crucial for the adherence of *C. neoformans* to the brain endothelial cells, since either eliminating hyaluronic acid or knocking down CD44 drastically reduced the adherence of cryptococcal cells to the endothelium (3, 17, 19). Consistent with these studies, we also found that a strain of *C. neoformans* that lacked hyaluronic acid was not able to effectively cross the HBMEC/D3 cell barrier (data not shown).

To examine whether the integrity of the HCMEC/D3 barrier was compromised by *C. neoformans*, we monitored transendothelial electrical resistance (TEER) of the HCMEC/D3 monolayer using an endometer (World Precision Instruments, Sarasota, FL) throughout the duration of the in vitro transcytosis assay in the presence or absence of cryptococcal cells (Fig. 1D). TEER values are routinely used to assess cell barrier integrity; however, these values are indicative only of a tight and functional barrier and are not conclusive (4, 8, 36). The final TEER value was obtained by subtracting the resistance of collagen-coated inserts from the resistance obtained in the presence of the endothelial cells. We found that the TEER values for the HCMEC/D3 monolayer in the presence of cryptococcal cells did not change over time and were not significantly different from those of control wells (Fig. 1D). Constant TEER values,
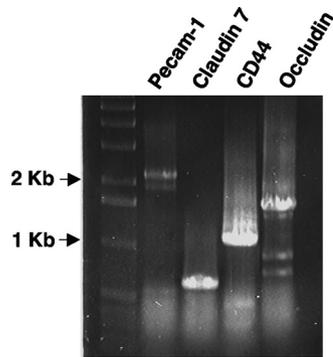
FIG. 2. The HCMEC/D3 cell line retains features of primary cells including the expression of pecam 1, CD44, claudin 7, and occludin. The transcripts of two key junctional markers, claudin 7 and occludin, and the glycoprotein receptor CD44 were confirmed using reverse transcriptase-PCR (RT-PCR) using RNA isolated from HCMEC/D3 cells alone. Pecam 1 was used here as an endothelial cell marker and represented a positive control for the RT-PCR. RNA was isolated from a culture of HCMEC/D3 (in the absence of *C. neoformans*) and purified according to the manufacturer's instructions (RNeasy kit; Qiagen, Valencia, CA). RT-PCR was performed with the following primers: for pecam 1, F-atgcagccgaggtgggcccac and R-ctaagttccatcaagg gagc; for claudin 7, F-atggccaattcgggcctgca and R-tcacacatactccttg-gaag; for CD44, F-atggacaagttttggtggca and R-ttacaccccaatcttcatg; and for occludin, F-atgtcatccaggcctcttga and R-ctatgttttctgtctatcat. The expected size of each amplicon was as follows: pecam 1, 2.1 kb; claudin 7, 640 bases; CD44, 1.1 kb; occludin, 1.5 kb. The PCR-generated amplicons were separated by using agarose gel electrophoresis and visualized by ethidium bromide staining. The RT-PCR conditions used were as follows: 30 min at 50°C, 15 min at 95°C, 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 4 min at 72°C (extension) (30 cycles), followed by 10 min at 72°C (extension).

which are indicative of a restrictive barrier, suggested that the integrity of the HCMEC/D3 barrier was maintained, and they supported the prevailing hypothesis that *C. neoformans* can cross the brain endothelium transcellularly without causing significant damage to the endothelial cells, since a major loss of barrier integrity would result in a reduction of TEER values over time (4, 8). Although the HCMEC/D3 monolayer had lower TEER values ($\sim$60 Ω) than those reported for primary brain endothelial cells ($\sim$150 Ω), it has been clearly demonstrated that this feature of the immortalized cell line is independent from its ability to form a highly restrictive barrier (36). In addition, the TEER values do decrease when the endothelial barrier is intentionally disrupted, indicating that TEER values of HCMEC/D3 cells (although low) do reflect an intact and functional barrier (36). Extensive characterization of HCMEC/D3 using permeability of low-molecular-weight compounds (including [^{14}C]sucrose, [^{14}C]diazepam, [^{14}C]morphine-6-glucuronide or [^{3}H]inulin, [^{3}H]imipramine, [^{3}H]prazosin, [^{3}H]colchicine, [^{3}H]vincristine, or fluorescein isothiocyanate-labeled dextran [4, 40, and 70 kDa]) has shown that this cell line exerts better restriction than primary cultures of bovine brain endothelial cells and resulted in permeability values similar to those obtained for primary cultures of human brain endothelium (4, 8, 36). This is likely because the HCMEC/D3 cell line retains all major functional features of primary brain endothelial cells, including the expression of tight junctional proteins at the cell borders (36). Accordingly, we confirmed the expression of transcripts in the HCMEC/D3 cell line (in the absence of cryptococcal cells) for two additional key junctional proteins, occludin 1 and claudin 7, and the surface glycoprotein receptor CD44 (Fig. 2). Taken together, these results indicate that the HCMEC/D3 cell line maintains an overall tight junction orga-
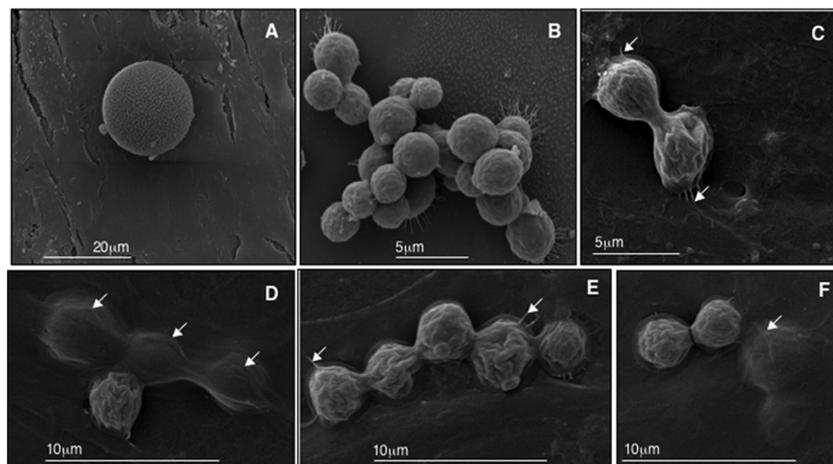

FIG. 3. *C. neoformans* cells can penetrate HCMEC/D3 endothelial cells and induce microvillus formations adjacent to the sites of invasion. (C to F) Endothelial cells grown on a transwell apparatus were infected with *C. neoformans* for 1 h and prepared for SEM. (A) HCMEC/D3 cells were incubated with glass beads for 1 h under conditions identical to those used with HCMEC/D3 cells in the presence of *C. neoformans.* (B) SEM images of cryptococcal cells in the absence of endothelial cells (but maintained in the EGM-2 medium) 1 h after they were added to collagen-coated transwell filters. Fibrous structures are likely collagen fibrils. (C) Pronounced microvillus formations on the in vitro barrier can be seen in the vicinity of yeast cells (arrows). (C and E) Cryptococcal cells that have been partially internalized are also shown in the SEM images (arrows). (D to F) Cryptococcal cells can be seen beneath the surface of the HCMEC/D3 monolayer (arrows). The images from SEM were obtained by following standard protocols described above. HCMEC/D3 cells were grown on transwell filters (8 μm; BD Biosciences) under the same conditions used for the transcytosis assays. Approximately 2 $\times$ 10^6 cells of a wild-type strain of *C. neoformans* (H99) were added to the top chamber for 1 h. The transwell filters were then washed with phosphate-buffered saline to remove nonspecific adhering yeast cells, fixed in Karnovsky's fixative, and sent to an on-campus SEM facility for sample processing (Electron Microscopy Lab, UC Davis). Glass beads (20 to 50 μm) were purchased from Sigma.

nization known to be present in the brain endothelium, which further supports the usefulness of HCMEC/D3 cells as an in vitro model of the BBB for cryptococcal studies.

Ultrastructural examination of brain endothelial cells infected with *C. neoformans* suggested that cryptococcal cells penetrated the HCMEC/D3 cell barrier, consistent with a transcellular movement of *C. neoformans* across the BBB (Fig. 3D and F) (4). Scanning electron microscopy (SEM) images revealed that HCMEC/D3 cells had undergone morphological changes that were observed only in the presence of cryptococcal cells and not when glass beads (20 to 50 μm; Sigma) were added to the endothelial cells (Fig. 3). Our results suggested that these changes in HCMEC/D3 cells were not due to a general stress reaction but rather occurred specifically in response to cryptococcal cells. Among the morphological changes that occurred was the production of microvilli on the surface of the endothelium (Fig. 3C to F, arrows). The microvilli could be seen attaching to and surrounding cryptococcal cells; however, microvilli were not observed anywhere near the glass beads (Fig. 3A). Interestingly, cryptococcal cells appeared healthier (more turgid and round) in the absence of the HCMEC/D3 cells (but still bathed in the EGM-2 medium), suggesting that perhaps the endothelium had created a hostile environment for *C. neoformans* (Fig. 3B). The SEM images also revealed the outline of cryptococcal cells just below the surface of the endothelial barrier, suggesting that these cryptococcal cells had been completely internalized by HCMEC/D3 cells, while others were only partially internalized (Fig. 3D and F, arrows). The morphological changes in the HBMEC/D3 cells reported here are consistent with similar changes observed in primary brain endothelial cells, further underscoring the tight similarity between the immortalized HCMEC/D3 cells used in this study and other primary cells (4, 36). Taken together, our results suggest that the HCMEC/D3 cell line is very well suited as a model of the BBB for resolving molecular mechanisms that determine how *C. neoformans* cells penetrate the brain endothelium and invade the CNS. This notion is supported by recent studies that have successfully used the HCMEC/D3 cell line as an in vitro BBB model to examine how bacterial and viral pathogens breach the BBB (10, 15).

In summary, we have shown that the recently developed immortalized brain endothelial cell line HCMEC/D3 is a suitable alternative to primary brain endothelial cells for in vitro studies of the BBB that will facilitate studies aimed at elucidating the invasion of *C. neoformans* into the CNS. Understanding its strong propensity for the CNS and its routes of entry will be essential for the identification of new targets required for future drug development aimed at treating CNS infection.

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