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# *Acanthamoeba* affects the integrity of human brain microvascular endothelial cells and degrades the tight junction proteins

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ABSTRACT

Haematogenous spread is a key step in the development of *Acanthamoeba* granulomatous encephalitis, however it is not clear how circulating amoebae cross the blood–brain barrier to enter the CNS to produce disease. Using the primary human brain microvascular endothelial cells (HBMEC), which constitute the blood–brain barrier, here it is shown that *Acanthamoeba* abolishes the HBMEC transendothelial electrical resistance. Using traversal assays, it was observed that *Acanthamoeba* crosses the HBMEC monolayers. The primary interactions of *Acanthamoeba* with the HBMEC resulted in increased protein tyrosine phosphorylations and the activation of RhoA, suggesting host–parasite cross-talk. Furthermore, Western blot assays revealed that *Acanthamoeba* degraded occludin and zonula occludens-1 proteins in a Rho kinase-dependent manner. Overall, these findings suggest that *Acanthamoeba* affects the integrity of the monolayer and traverses the HBMEC by targeting the tight junction proteins.



## 1. Introduction

*Acanthamoeba* granulomatous encephalitis is a rare infection that almost always proves fatal. The majority of *Acanthamoeba* encephalitis cases occur in immunocompromised individuals. This infection is of concern in view of (i) an increasing immunocompromised population, and (ii) individuals undergoing immunosuppressive therapy and excessive use of steroids. Perhaps the most distressing aspect is the limited availability of effective treatment against *Acanthamoeba* granulomatous encephalitis, which at present has a case fatality rate of more than 90%. The gross pathology of the autopsied brain often shows severe oedema and haemorrhagic necrosis and encephalitis (reviewed in Marciano-Cabral and Cabral 2003; Khan, 2006, 2008; Visvesvara et al., 2007).

It is widely accepted that the route of entry for *Acanthamoeba* includes the respiratory tract, leading to amoebae invasion of the alveolar blood vessels, followed by haematogenous spread. Skin lesions may provide direct entry into the bloodstream, bypassing the lower respiratory tract. *Acanthamoeba* entry into the CNS most likely occurs through the blood–brain barrier, which is highly selective in regulating the entry of microbes and/or molecules. The olfactory neuroepithelium route (i.e. invasion of the olfactory part of the nasal epithelium and migration along nerve fibres, followed by invasion of the olfactory bulb) provides another route of entry into the CNS and has been studied in experimental models (Culbertson et al., 1959; Cerva, 1967; Martinez, 1985, 1991; Martinez and Visvesvara, 1997). It is worth noting that the olfactory part of the nasal mucosa in the rat (rodents) is 50% of the total surface area of the nose, whilst in humans, the olfactory part of the nasal mucosa is only 3–8% (Illum, 2000; Graff and Pollack, 2005). The smaller fraction of nasal mucosa associated with the olfactory epithelium in humans can be associated with a lower likelihood of this route leading to human disease. Furthermore, the widespread distribution of lesions in the brain, as observed in *Acanthamoeba* granulomatous encephalitis patients, would fit better with haematogenous spread than olfactory access. *Acanthamoeba* entry into the CNS most likely occurs through the endothelial lining of the cerebral capillaries (Martinez, 1985, 1991; Martinez and Visvesvara, 1997). Hematoxylin–eosin-stained sections of the brain tissue of *Acanthamoeba* granulomatous encephalitis patients exhibit large numbers of amoebae in the perivascular space, indicating the involvement of the cerebral capillaries as the sites of entry into the CNS. Affected tissues other than the CNS may include s.c. tissue, skin, liver, lungs, kidneys, adrenals, pancreas, prostate, lymph nodes and bone marrow, which further suggest haematogenous

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spread. Overall, these findings suggest that unlike *Naegleria fowleri* which uses the olfactory neuroepithelium route, *Acanthamoeba* invades the CNS via the blood–brain barrier. The mechanisms associated with *Acanthamoeba* traversal of the blood–brain barrier are not clear. The present study determined the effects of *Acanthamoeba* on the human brain microvascular endothelial cells that constitute the blood–brain barrier. The findings revealed that *Acanthamoeba* induced blood–brain barrier perturbations by targeting the tight junction proteins.

## 2. Materials and methods

### 2.1. Culture of Acanthamoeba

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated. For the present study, *Acanthamoeba castellanii* belonging to the T4 genotype was obtained from the American Type Culture Collection (ATCC 50492). As, based on the 18S rRNA gene sequencing, most of the clinical isolates of *Acanthamoeba* (from keratitis, encephalitis and cutaneous infections) as well as environmental isolates have been typed as the T4 genotype, the aforementioned isolate was used as a representative of the T4 genotype. *Acanthamoeba* cultures were grown without shaking in 15-ml of PYG medium (proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)) in T-75 tissue culture flasks at 30 °C as previously described (Sissons et al., 2005) and media refreshed 17–20 h prior to experiments. *Acanthamoeba* adhering to flasks represented trophozoite forms and were used for all assays.

### 2.2. Human brain microvascular endothelial cell cultures

The primary human brain microvascular endothelial cells (HBMEC) were used as previously described (Stins et al., 1997; Alsam et al., 2003). Briefly, small fragments of cerebral cortex, derived from individuals who had undergone surgical resections for seizure disorder at Johns Hopkins University School of Medicine (USA) were used for HBMEC isolation following approval from the University Ethics Committee. The primary endothelial cells were purified by fluorescent activated cell sorting, and their purity tested using endothelial markers such as expression of F-VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (DiI-AcLDL) as previously described (Stins et al., 1997; Alsam et al., 2003) and resulted in >99% pure endothelial cultures. The HBMEC were routinely grown on rat-tail collagen-coated dishes in complete medium (RPMI-1640 containing 10% heat-inactivated FBS, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino acids and vitamins) (Invitrogen, Paisley, England).

### 2.3. Traversal assays

The HBMEC were seeded into the upper chamber of collagen-coated Transwells, in a 24-well Transwell plate (Corning Costar, Corning Ltd., Hemel Hempstead, England), and grown to confluency for at least 2 days in HBMEC culture media as described previously (Stins et al., 2001; Alsam et al., 2005). Following this incubation, the media were replaced with fresh culture media containing 500 nM hydrocortisone and cultured for an additional 3–5 days. The integrity of monolayers was monitored by measuring transendothelial electrical resistance (TEER) using a tissue-resistant measurement chamber and a voltohmmeter (EVOM, World Precision Instruments). The HBMEC monolayers exhibiting TEER of more than 200 $\Omega/cm^2$ were subsequently used for traversal assays. To determine the effects of *Acanthamoeba* on TEER, amoebae were added to the upper chamber of the Transwells ($5 \times 10^5$ amoebae; total volume 200 μl; counted using a haemocytometer) and incubated for up to 8 h. Following this incubation, the integrity of the HBMEC monolayer was determined by measuring TEER as described above. In addition, the effects of *Acanthamoeba* conditioned medium (CM) on HBMEC monolayer integrity were determined. The CM was produced by incubating *Acanthamoeba* ($5 \times 10^6$ amoebae/ml) in RPMI-1640 for 17–20 h. Following this incubation, cell-free CM were collected by centrifugation and used for traversal assays.

To evaluate the ability of *Acanthamoeba* to cross the HBMEC monolayer, amoebae were added to the upper chamber and allowed to cross the HBMEC monolayer for up to 8 h in the presence or absence of a rho kinase inhibitor, Y27632 (Merck Chemicals, UK). Subsequently, 100 μl samples were taken from the bottom chamber and the number of amoebae determined by haemocytometer counting.

### 2.4. Western blot assays

To determine the effects of *Acanthamoeba* on the HBMEC monolayer tight junction proteins, Western blot assays were performed. Briefly, HBMEC were grown to confluency in six-well plates and monolayers were incubated with live amoebae ($5 \times 10^6$ amoebae/ml) or their CM for 90 min in the presence or absence of a rho kinase inhibitor, Y27632. The CM was produced by incubating *Acanthamoeba* ($5 \times 10^6$ amoebae/ml) in RPMI-1640 for 17–20 h as indicated above. Following this incubation, CM were collected by centrifugation and incubated with the HBMEC monolayer. For controls, HBMEC alone or HBMEC incubated with methanol-fixed amoebae were used. Following incubation, cells were lysed with RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 0.1% SDS, 0.5% Na-deoxycholate, 10 mM Na-pyrophosphate, 25 mM β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM $Na_3VO_4$, 50 mM NaF, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Samples were centrifuged (10,000*g*, 4 °C, 6 min), and supernatant collected for protein quantification (Bio-Rad protein detection kit). Equal amounts of proteins were electrophoresed using 10% SDS–PAGE under denaturing conditions. Proteins were transferred onto nitrocellulose membranes and blocked in 4% blocking buffer (blotting grade non-fat milk solution; Bio-Rad, Hemel Hempstead, England). The membranes were blotted with anti-zonula occludens (ZO)-1, and anti-occludin antibodies (Invitrogen), in the blocking buffer overnight at 4 °C, with gentle shaking. The next day, the membranes were washed and subsequently incubated (60 min, 4 °C) with appropriate horseradish peroxidase-linked secondary antibodies (Cell Signalling Tech, Beverly, MA, USA). Protein bands were visualised using an enhanced chemiluminescent detection kit (Amersham Biosciences, England).

### 2.5. GST-rhotekin assays

To determine effects of *Acanthamoeba* on the activation of HBMEC RhoA, monolayers were incubated with *Acanthamoeba* ($5 \times 10^6$ amoebae) for 90 min, and lysates prepared using RIPA buffer and protein quantified as described previously (Khan et al., 2002). An equal amount of protein (500 μg to 1 mg) was incubated with GST-rhotekin (Upstate Biotech, Lake Placid, NY, USA) for 45 min at 4 °C to collect active forms of RhoA, i.e. GTP-RhoA. The protein complex was washed with RIPA and resolved by 10% SDS–PAGE and transferred to nitrocellulose membrane. The blots were blocked with TBST (25 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween 20) containing 4% skimmed milk for 60 min at 22 °C. After blocking, the membranes were incubated overnight (4 °C) with mouse monoclonal antibody against RhoA (Santa Cruz Bio-