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Acanthamoeba interactions with the blood–brain barrier under dynamic fluid flow

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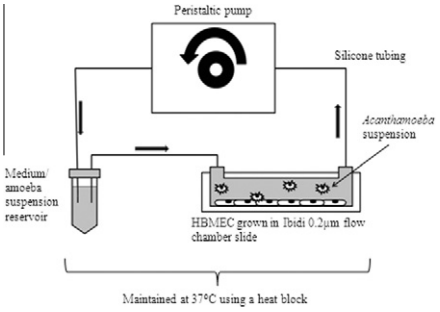
HIGHLIGHTS

- Novel *in vitro* method of modelling dynamic blood–brain barrier interactions with extracellular parasites.
- Discrepancy between static culture and flow system binding rates.
- Host cell monolayer disruption not correlated to binding under flow conditions.
- Flow system highlights the importance of the *Acanthamoeba* mannose binding protein in pathogenesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Acanthamoeba granulomatous encephalitis (AGE), caused by *Acanthamoeba castellanii*, is a fatal infection of immunocompromised individuals. The pathogenesis of blood–brain barrier (BBB) breach remains unknown. Using a novel *in vitro* BBB infection model under flow conditions, demonstrates that increases in flow rates lead to decreased binding of *A. castellanii* to host cells. This is a distinct departure from previous findings under static conditions. However, similarly to static conditions binding of *A. castellanii* to host cells is host mannose dependent. Disruption of the host cell monolayer was independent of amoeba binding, but dependent on secreted serine proteases. For the first time we report the binding dynamics of *A. castellanii* under physiological conditions, showing that BBB disruption is not directly linked to binding, instead it is reliant on secreted proteases. Our results offer a platform on which therapies designed at modulating physiological parameters can improve the outcome of infection with *A. castellanii*.

1. Introduction

Free-living amoeba of the genus *Acanthamoeba* are found commonly in a variety of both soil and aquatic environments worldwide. Their lifecycle alternates between two states: that of an active, proliferating trophozoite when external environmental conditions are favourable and a dormant cyst form when conditions are hostile. In this latter form *Acanthamoeba* are highly resistant to desiccation, pH, temperature and the external chemical environment (Aksozek et al., 2002; Coulon et al., 2010; Lloyd et al., 2001).

Acanthamoeba species also exhibit the ability to cause disease in animals and man with two distinct clinical presentations; sight-threatening keratitis and potentially fatal amoebic granulomatous encephalitis (AGE). The latter condition is thought to occur when amoebae spread haematogenously from a cutaneous or mucus membrane infection (Culbertson et al., 1959; Culbertson et al., 1958; Martinez, 1991). Following haemotogenous spread amoebae then cross the blood–brain barrier (BBB) and enter neural tissue where they cause haemorrhagic necrosis, severe encephalitis and form granulomatous lesions. Prognosis for the condition is typically poor due to difficulties in reaching a definitive diagnosis combined with a lack of effective therapies and as such AGE infections frequently result in death (Bloch and Schuster, 2005; Sarica et al.,

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2009; Velho et al., 2003). Impaired immunity is thought to be a particular risk factor for development of AGE, and as such there is a considerable proportion of the global population at risk of developing the infection, particularly amongst areas of endemic HIV infection (Di Gregorio et al., 1992; Gardner et al., 1991; Kumar et al., 2007; Sison et al., 1995; Wiley et al., 1987).

A key event in the establishment of AGE is penetration of the blood–brain barrier. Frameworks for understanding how this occurs have been elaborated by previous authors, revealing contributions from direct binding proteins and protease secretions (Khan and Siddiqui, 2009; Sissons et al., 2006, 2004). However the scope of these studies is restricted by the use of static models of *Acanthamoeba* interaction with the BBB. The brain microcirculation is a dynamic system and varies significantly in response to metabolic demands, so removed from this context it is likely that current methods do not provide an accurate representation of infection *in vivo*. In light of this we have developed an *in vitro* system using human brain microvascular endothelial cells (HBMEC) grown in flow chamber slides, and exposed these under flow conditions to *A. castellanii* trophozoites (T4 genotype) or to collected protease secretions. Our data indicate a role for variable cerebral blood flow rate in adhesion of *A. castellanii* trophozoites to brain endothelial cells and implicate serine proteases as a major cause of BBB disruption. This provides important insights into the initiation of BBB traversal by *Acanthamoeba* and may help to refine our understanding of AGE pathogenesis.

2. Materials and methods

2.1. Parasite and tissue culture

Amoeba culture: *A. castellanii* from the T4 genotype (ATCC 50492) were maintained in axenic peptone-yeast-glucose (PYG) growth media containing 0.75% proteose peptone, 0.75% yeast extract and 1.5% glucose as previously described (Alsam et al., 2003). Approximately 18 h prior to experiments, cultures were fed with fresh media to maintain >95% trophozoites. To collect amoeba suspensions flasks were chilled to detach adherent trophozoites then removed from PYG medium by centrifugation at 1000*g* for 5 min. The pellet was washed once in RPMI to remove any trace PYG, then resuspended in RPMI to a density of 2×10^5/ml.

Acanthamoeba conditioned medium (ACM): Cultures of amoeba were grown to confluence before being washed twice in RPMI to remove PYG traces. Cultures were then incubated in 50 ml RPMI for three days. Medium was harvested by centrifugation to pellet amoeba cells in suspension followed by passage through a 0.22 μm sterile filter.

HBMEC culture: Primary HBMEC originated from individuals who had undergone neurosurgery at Johns Hopkins University School of Medicine (USA) as described in Khan and Siddiqui (2009). Isolation followed methods detailed by Alsam et al. (2003) and Stins et al. (1997), and purified cultures obtained by fluorescent-activated cell sorting (FACS) tested positive for endothelial markers Factor-VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (Alsam et al., 2003; Khan and Siddiqui, 2009; Stins et al., 1997). HBMEC were maintained in media containing 20% Foetal Bovine Serum, 1% Penicillin/Streptomycin, 1% L-glutamine, 1% non-essential amino acids, 1% Minimum Essential Medium vitamins and 1% sodium pyruvate at 37 °C + 5% CO_2. Under these conditions HBMEC have been shown to retain trans-endothelial electrical resistance (TEER) of >200 mΩ and exhibit barrier function against bovine serum albumin (BSA) (Alsam et al., 2005b). Cells were used between passages 11–20. For flow slide culture 2.5×10^5 cells from fully confluent flasks were seeded into Ibidi 0.2 Luer chamber slides in standard growth medium. Media was changed daily to maintain steady growth and HBMEC reached confluence after 2–4 days.

Infection with A. castellanii: Flow chambers were washed in RPMI to remove traces of growth media and then attached to 1.6 mm tubing primed with amoeba suspension using Ibidi adaptors and recirculated into a stock solution. A pre-calibrated flow speed setting was selected and the system acclimatised for 10 min before initiating the experiment. Stock solutions and flow slides were maintained at 37 °C with a heat block, and the stock was regularly agitated to prevent amoeba settling out of solution. A schematic illustration of the flow system is given in supplementary Fig. 1.

In order to compare readings taken under flow conditions with standard static conditions, parallel experiments were set up using methods previously described (Alsam et al., 2003). Briefly, 2.5×10^5 cells were seeded into wells of 24-well plates in standard growth media and left to adhere and grow to confluence overnight. Monolayers were washed in RPMI to remove media traces, then infected with 1 ml of 2×10^5/ml amoebae and kept at 37 °C for up to 3 hours.

2.2. Binding and disruption measurements

Binding was assessed by triplicate counts taken from the stock solution at time points during, or at the termination of, the experiment. Counts of the number of unbound amoeba were converted to the number of adherent amoeba by subtraction from the amoeba density, and expressed as a percentage. To test the involvement of sugar residues in binding amoeba suspensions were treated with either 50 mM D-mannose, 50 mM D-glucose or were left untreated. Amoeba were incubated with sugars for 30 min at 37 °C prior to infecting monolayers.

2.3. Use of Acanthamoeba conditioned medium (ACM) in flow system

Medium was diluted 1 in 2 in RPMI and either treated with 5 mM phenylmethylsulphonyl fluoride (PMSF), a broad-spectrum serine protease inhibitor or left untreated. PMSF-treated RPMI was used as a control. Prior to the experiment, media was left to incubate for 90 min at 37 °C to minimise the effect of active PMSF on cells. ACM was then circulated through the flow system or added to static plates in place of amoeba suspension.

2.4. Monolayer disruption

At time points during, or at the conclusion of the experiment, triplicate images of monolayers were taken using a Leica DMB5000B microscope, using inbuilt auto-exposure and white balance settings. Open-source Image J software (available from http://rsbweb.nih.gov/ij/) was then used to quantify disruption using the following method. Firstly a grid was superimposed over the image; 1600 pixels per square to give a total of 5000 squares. Five representative blocks of 196 squares per block (14×14) were then selected, and each individual square in a block scored as disrupted if cell coverage was $\leqslant$50%. This score was then expressed as a percentage of the total 980 squares counted. A schematic representation of the method is given in supplementary Fig. 2.

2.5. Statistical analysis

Analyses were performed using GraphPad Prism software version 5.01. Data was analysed using a combination of analysis of variance (ANOVA) and regression models. Alpha level was set at 0.05 for all tests.