

# Next generation of non-mammalian blood-brain barrier models to study parasitic infections of the central nervous system

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Transmigration of neuropathogens across the blood-brain barrier is a key step in the development of central nervous system infections, making it a prime target for drug development. The ability of neuropathogens to traverse the blood-brain barrier continues to inspire researchers to understand the specific strategies and molecular mechanisms that allow them to enter the brain. The availability of models of the blood-brain barrier that closely mimic the situation in vivo offers unprecedented opportunities for the development of novel therapeutics.

## Introduction

Traversal of neuropathogens from the lumen of cerebral microvessels to the brain is a pre-requisite in the development of central nervous system (CNS) infections. Thus the transmigration of neuroparasites across the blood-brain barrier (BBB) in disease states makes it a prime target for drug development. The model systems that have been used to study this process at the molecular level do not closely mimic the situation in vivo. Adhesion and cytopathogenicity data, as well as measures of monolayer disruption are generally obtained from static cultures which provide useful information at an early stage but cannot mirror ordinary blood flow. Thus, there is a need to develop next generation models of BBB with expanded physiological relevance and ethical acceptability. In the real situation, transmigration of pathogens into the CNS takes place within the cerebral microvessels under dynamic fluid flow, which affects many biological properties.<sup>1</sup> Using brain microvascular endothelial cells (BMEC), which constitute the blood-brain barrier, researchers are studying parasite-BBB interactions leading to parasite traversal of the blood-brain barrier.<sup>2-8</sup> Here, we describe the in vitro models of the BBB and how they can be improved to mimic the microvessels under dynamic fluid flow. In addition, the use of insects such as locusts is discussed as an in vivo model of the BBB to study transmigration of neuropathogens into the CNS.

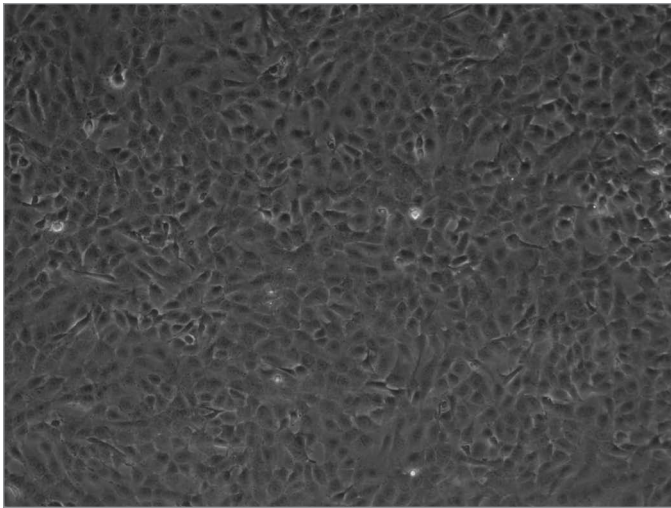
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## In Vitro Model of the Blood-Brain Barrier for Biochemical/Molecular Studies

The human BMEC that constitute the BBB are important tools for rapid identification of molecular determinants involved in the parasite-BBB interactions (Fig. 1). The BMEC derived from human brain tissue are positive for markers such as factor VIII, carbonic anhydrase, Ulex Europaeus Agglutinin I, gamma-glutamyl transpeptidase and uptake acetylated low-density lipoprotein indicating their brain endothelial origin.<sup>9,10</sup> The human BMEC are joined together by the presence of tight junction proteins such as occludins, claudins, junction adhesion molecules, endothelial cell-selective adhesion molecules and zonula occludens-1 (ZO-1), ZO-2, ZO-3 making it a highly selective barrier compared with the peripheral endothelium.<sup>11</sup> The tight junction proteins are primarily responsible for the barrier function, preventing entry of blood contents into the CNS.<sup>11</sup> The human BMEC can be routinely grown on rat-tail collagen-coated dishes in RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 µg/ml), non-essential amino acids and vitamins.<sup>9,10</sup> This model can be used to identify molecular determinants (adhesins and receptors) involved in parasite-host cell interactions, in particular for biochemical studies where there is a requirement of a large number of cells. The in vitro BBB model system has already provided novel insights into the molecular mechanisms of translocation of BBB by many microbial pathogens<sup>2-8</sup> and will continue to facilitate the study of pathogen-BBB interactions.

## In Vitro Model of the Blood-Brain Barrier for Parasite Transmigration Studies

The Transwell in vitro model is created by cultivating human BMEC in the upper chamber of collagen-coated Transwells polycarbonate tissue culture inserts (Corning Costar, Corning Ltd.) (Figs. 1 and 2), and grown for at least 2 d in BMEC culture media. Next, the media are replaced with fresh culture media containing 500 nM hydrocortisone and cultured for an additional 3–6 d. Culture medium containing hydrocortisone is changed every 2 d and confluency determined by light microscopy. Under



**Figure 1.** Phase contrast image of primary human brain microvascular endothelial cells positive for markers such as factor VIII, carbonic anhydrase, Ulex Europaeus Agglutinin I, gamma-glutamyl transpeptidase, and uptake acetylated low-density lipoprotein as well as presence of tight junctions indicating their brain endothelial origin.

these conditions, the BMEC monolayer exhibits tight junction formation and polarization and develops TEER of more than 200 ohm/cm<sup>2</sup> using a tissue-resistant measurement chamber and a voltohmmeter (EVOM, World Precision Instruments).<sup>8,12</sup> For transmigration studies, parasites are added in the upper chamber and TEER changes can be monitored. In addition, to investigate the ability of parasites to alter the human BMEC monolayer integrity, parasites can be added to the upper chamber and allowed to cross the monolayer. Subsequently, samples are taken from the bottom chamber and the number of parasites can simply be determined by hemocytometer counting. As well as studying disease pathogenesis, this is a potentially useful model to test novel drug therapies, *in vitro* and the role of potential adhesins/receptors involved in parasite-host cell interactions leading to the blood-brain barrier traversal. Static systems for investigating pathogenesis of CNS parasites have been widely used and yielded valuable discoveries in the effort to better understand parasite traversal of the BBB and will continue to facilitate the study of pathogen invasion of the CNS.

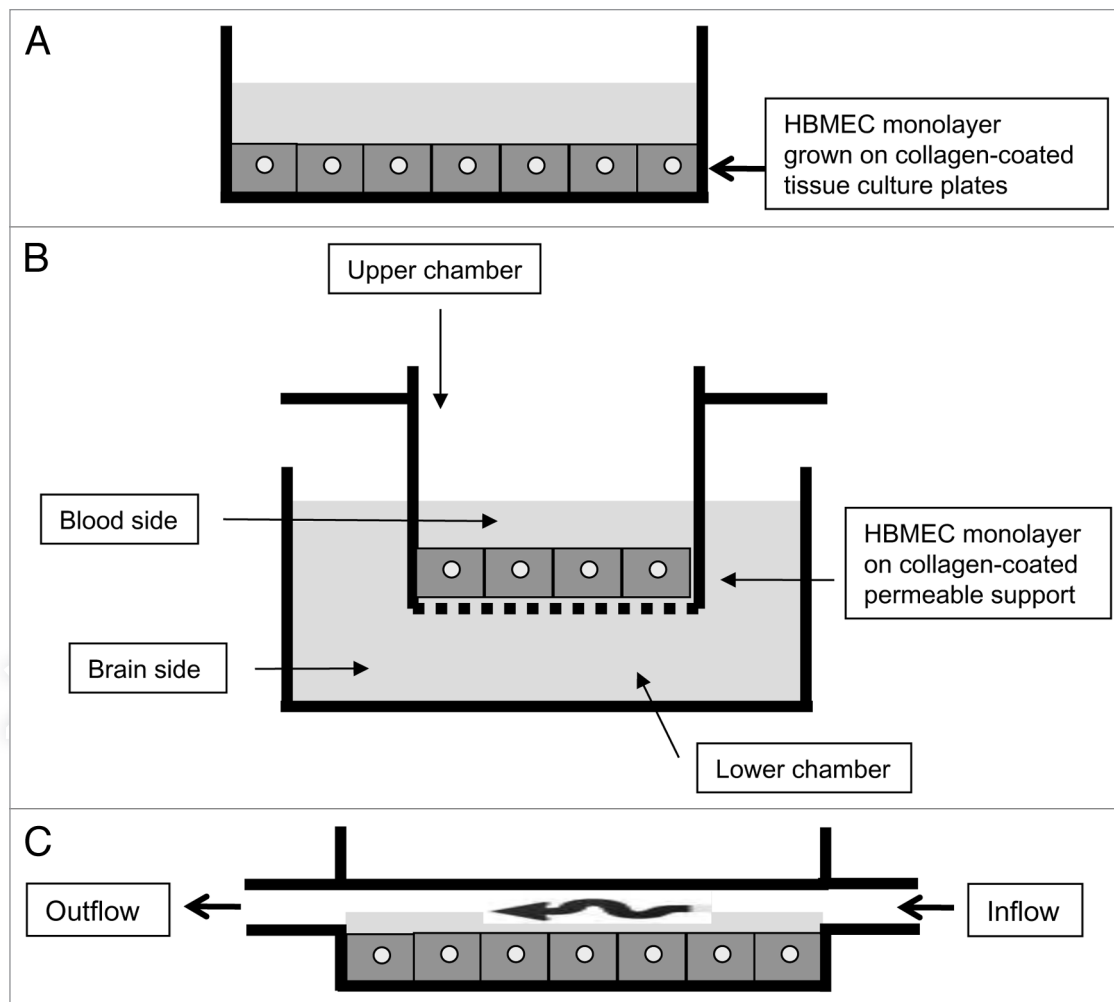
### **In Vitro Model of the Blood-Brain Barrier under Physiological Flow**

Transmigration of parasites from the lumen of cerebral microvessels to the CNS takes place under dynamic fluid flow, which affects many biological properties.<sup>1</sup> Static conditions *in vitro* limit the usefulness of the aforementioned models and so a novel dynamic flow system has been established to create a closer analog to the infection *in vivo* (Fig. 3). Briefly, sterile tissue culture grade Luer 0.2 μm microslides (Figs. 2 and 3) are seeded with the human BMEC and allowed to grow to confluency over a period of several days. Briefly, 50 μl of cell suspension containing 2.5 × 10<sup>5</sup> cells is pipetted into the anterior chamber of a

pre-warmed slide. Both anterior and posterior chambers are filled with 60 μl of growth medium and slide incubated at 37°C in a 5% CO<sub>2</sub> and media refreshed daily. At this cell density, BMEC formed confluent monolayers within 3 d. On the day of testing, chambers are brim-filled with fresh media to prevent the entry of air bubbles and attached to a piping system which had also been pre-filled with media, passing through a peristaltic pump (Fig. 3). Prior to initiation of the experiment, microslides are flushed with RPMI growth medium for 5 min, with media drawn from a stock tube through the system and into a waste beaker. For the experimental runs, flow is re-circulated through the initial stock to avoid it running dry and exposing the cells to air bubbles. This can be kept at 37°C in a water bath. The novelty of this approach stems from an ability to alter flow rates using peristaltic pump to physiologically relevant levels and produce semi real-time observations of immediate binding and monolayer disruption effects produced by pathogens and their conditioned medium. In our studies, a flow slide that had been seeded at optimal density and grown to confluence for three days was attached to a peristaltic flow system and incubated with warmed RPMI 1640 at a flow rate of 0.1–3 ml/min without affecting the BMEC integrity. During the experiment, the microslide section of the system is placed under an inverted microscope to observe cells. Under these conditions, human BMEC retain normal monolayer characteristics when subjected to flow. Such a model offers physiological relevance by emulating vascular flow and shear stress conditions while pulsatile flow, temperature control and gas exchange provide additional capabilities. It will be instrumental in identifying molecular mechanisms and will yield and/or test potential targets for therapeutic intervention.

### **Non-Vertebrate Model of BBB for the In Vivo Study of Neuropathogens**

Despite the fact that the genome of many neuropathogens has been sequenced, only a limited number of virulence-associated genes are identified. This is partly due to a total reliance upon mammalian models to study neuropathogenesis. The vertebrate models are physiologically relevant, are expensive, involve challenging mechanical manipulations, anatomical complexities and long reproductive cycles and require legislative adherence. Although invertebrate host animals do not reproduce all aspects of vertebrate infection, specific scientific questions can be addressed using an appropriate selection of a non-mammalian model system. For example, mechanisms of phagocytosis can be studied using amoebae as a host organism, while innate immune responses can be studied using insects. More recently, studies have shown the use of locusts as a valuable tool to understand the mechanisms of parasite penetration across the BBB and discriminate molecules participating from both sides of the host-bacterium interaction.<sup>13-15</sup> Parasites are injected into the hemocoel of the locust abdomen and their sickness behavior, disease pathogenesis, BBB integrity and mortality can be studied.<sup>13,15</sup> For brain dissections, the left side of head is removed by a sagittal cut and the brain dissected out using fine forceps for further studies (Fig. 4).<sup>13</sup> In this model, progress of infection is monitored by

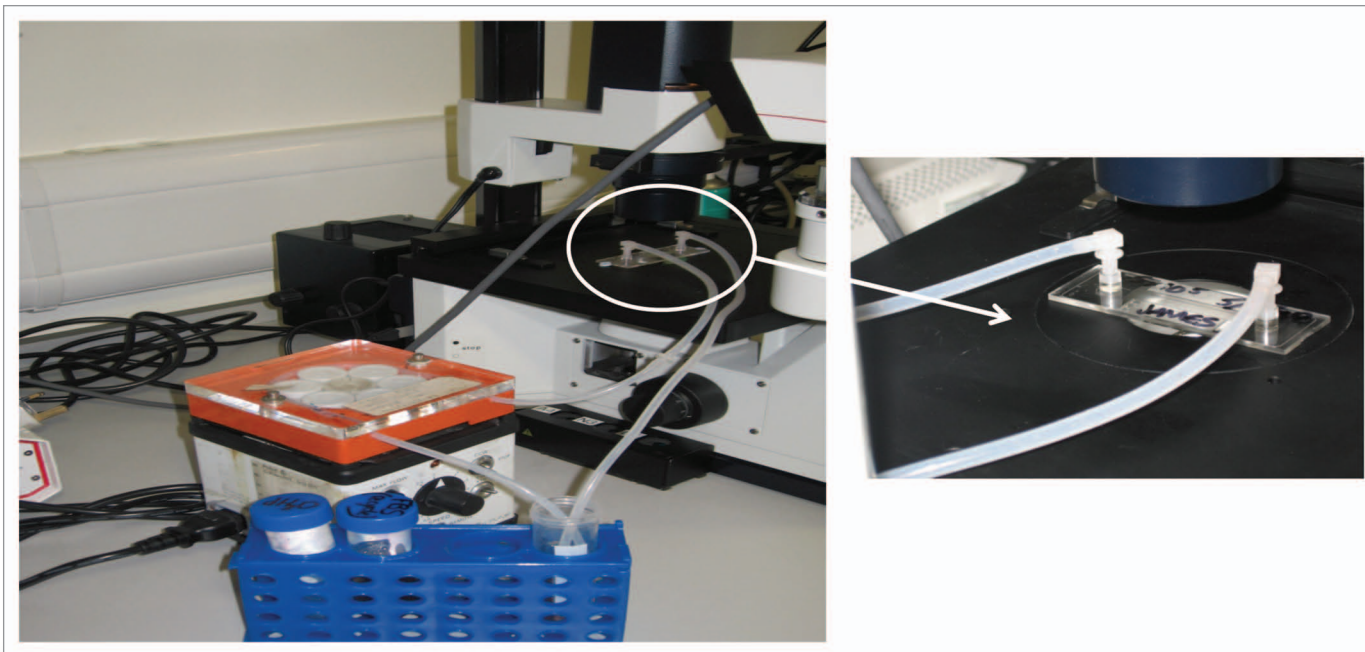


**Figure 2.** (A) The human BMEC can be grown to confluency in tissue culture plates in large numbers that can be used subsequently for biochemical/molecular studies. (B) BMEC are grown as a monolayer on Transwell inserts. Parasites can be added in the upper chamber (blood-side). After various intervals of time, the contents of the lower chamber (brain-side) can be removed to determine parasite translocation of the blood-brain barrier. (C) BMEC are grown in microslides and parasite interactions can be studied under physiological flow conditions using a peristaltic pump. The microslide is placed under an inverted microscope to observe parasite-host interactions in real time.

the death of the host making it a particularly useful model as there is an explicit endpoint as opposed to a complex phenotype in higher animals.

Although the BBB in insects has been well studied in relation to ion balance, for the first time we showed its penetration by bacteria (neuropathogenic *Escherichia coli* K1)<sup>13</sup> and pathogenic protist (*Acanthamoeba*).<sup>14,15</sup> As the BBB of both insects and mammals are formed by a single layer of cells (glial cells and endothelial cells respectively), joined by tight junctions, our scientific hypothesis was that in insects, as in mammals, neuropathogens would cross the BBB. Thus, at the molecular level there will be similarities between the mechanisms by which pathogens such as *E. coli* K1 and *Acanthamoeba* invade the CNS of mammals and of locusts. Our recently published findings supported this speculation.<sup>13,16</sup> It is interesting to note that *Acanthamoeba* invades the locust brains by modulating the integrity of the locust blood-brain barrier,<sup>15</sup> a finding that is consistent with *Acanthamoeba* traversal of human brain

microvascular endothelial cells.<sup>8</sup> These studies were carefully executed and allowed direct comparison with previous data obtained using mammalian-based models. We believe that the use of a more technically convenient locust model could be used for the initial screening and identification of novel virulence factors, which will provide useful leads for the rationale development and evaluation of therapeutic interventions. Although *Drosophila* has emerged as an important animal model that provides genetic tractability, the use of locust is advantageous, in that, locusts are fairly large, easy to handle, and relatively large volumes can be injected and/or retrieved, and the locust brains can be dissected out with ease for further analyses. In contrast, challenges of mechanical manipulation associated with *Drosophila* such as systemic injections and tissue dissections may affect the study. The similarities between insects' and vertebrates' BBB and associated homologous molecular determinants<sup>17</sup> suggest that insects make useful models to study the neuropathogenesis of CNS pathogens. For example,



**Figure 3.** The human BMEC are grown in collagen-coated microslides. Once confluent, slide is attached to a peristaltic flow system and incubated with warm RPMI-1640 at a variable flow rate. Parasites are added in the flow tube (50 ml centrifuge tube). Parasite-host cell interactions are examined microscopically in real time under dynamic fluid flow.

the understanding of two parallel signaling cascades, the Toll and Imd pathways, involved in recognition of pathogens, come from insects.<sup>18</sup> The Toll pathway is conserved between insects and mammals and it is essential for inflammatory cytokine production in mammals. As long as the unique strengths and limitations associated with the selection of an appropriate model system are recognized, it is reasonable to suggest that interactions of pathogens with insects as animal hosts will continue to provide useful insights into the study of microbial pathogenesis. The use of insect hosts in genetic screens that require a large number of animals make them an attractive invertebrate model. In addition, the use of invertebrates in high throughput in vivo assays make them important tools that will go some way toward replacing the use of vertebrate animals in research at an early stage, and will reduce the numbers of mammals required overall. The proposed invertebrate models are a timely response to the

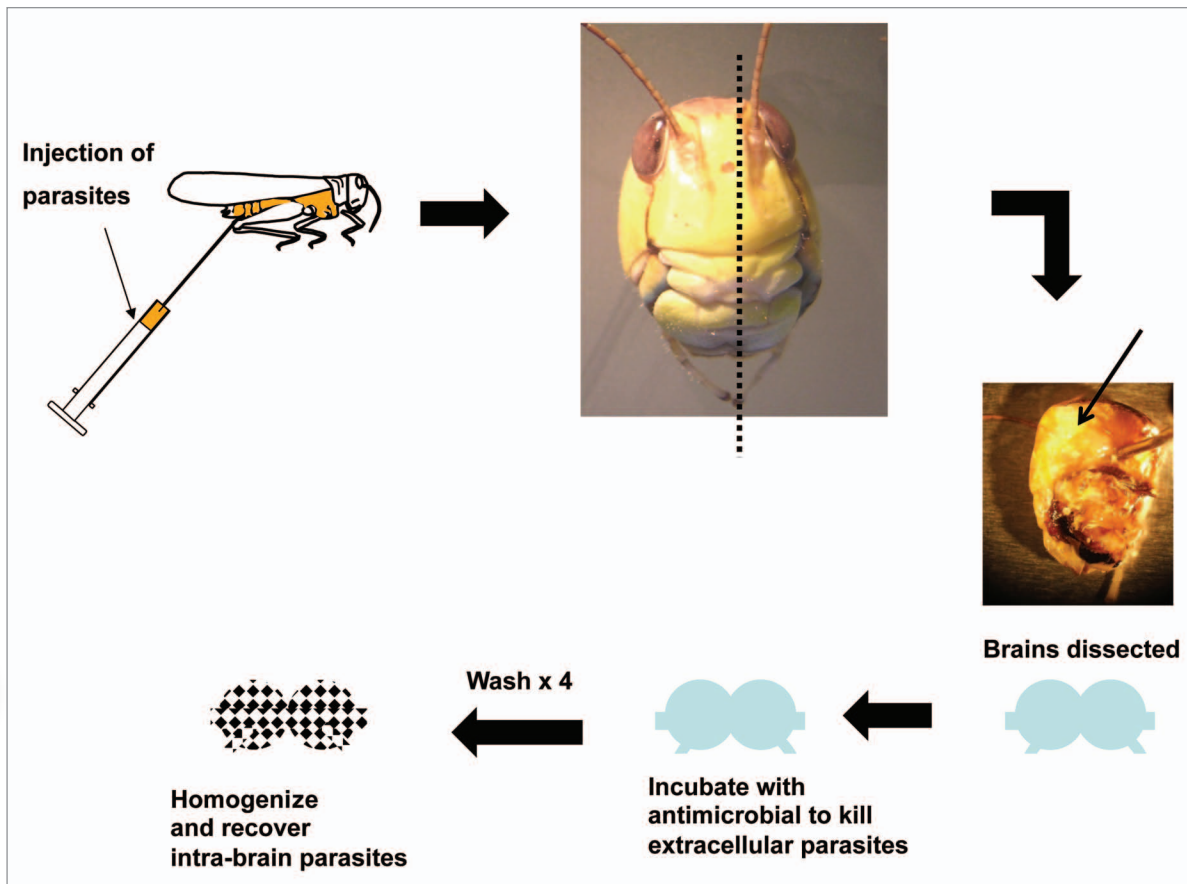
expressed desires of the public and governments to replace and reduce the use of mammals in research. Based on these findings, it is hoped that other scientists will consider using commonly available insects such as locusts as tractable models for studying disease pathogenesis in vivo and human BMEC under physiological flow in vitro. Future studies will continue to explore the development of better model systems that are needed for studying the pathogenicity of microbial organisms.

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**Figure 4.** Locusts as an in vivo model to study parasitic infections of the central nervous system. Parasites are injected into locusts' abdomen and brain dissected out to study microbial invasion of the central nervous system. Briefly, locusts are killed and head capsules are removed from the body. Left side of the head is removed by a sagittal cut and brains are dissected out using forceps to demonstrate the presence of neuropathogens.

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