Pathogenesis of Cerebral Malaria: Inflammation, Cytoadherence and Recent Experimental Data for Humans

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Abstract: This study establishes that HRPII is a Plasmodium falciparum virulence factor that triggers an innate immune inflammatory response in vascular endothelium and contributes to cerebral malaria by compromising the integrity of the blood-brain barrier. Human malaria is caused by five species of Plasmodium. Of these, P. falciparum is the deadliest and is the only species that causes cerebral malaria (CM). CM is a disease of the vascular endothelium considered by parasite sequestration, increased inflammatory cytokine production, vascular leakage and leukocyte infiltration. A distinguishing feature of P. falciparum infection is the parasite’s production and secretion of histidine-rich protein II (HRPII). HRPII accumulates to high concentrations (up to 100 g/ml) in serum, which correlates with disease severity. Using a cellular model of the blood-brain barrier, it demonstrate that HRPII activates the innate immune system in human cerebral microvascular endothelial cells, resulting in redistribution of tight junction proteins and compromise of barrier integrity. Intravenous infusion of HRPII induced vascular leakage in the cerebellum and cortex of mice and increased early mortality in a P. berghei ANKA experimental cerebral malaria model. Analogously, transgenic P. berghei expressing falciparum HRPII produced more severe disease than wild-type or control P. berghei. HRPII induced endothelial expression of adhesion receptors used by plasmodium parasites, suggesting that this protein also contributes to pathogenesis by enhancing parasite cytoadherence and thereby avoiding splenic destruction.

Key words: Epidemiology, Pathophysiology, Cerebral Malaria, Mouse Models for Cerebral Malaria, HRPII, Blood Brain Barrier, Vascular endothelium, Inflammasome

Epidemiology and Pathophysiology

Malaria is a mosquito transmitted parasite illness present in some of the most economically disadvantaged populations world-wide. According to recent WHO estimates roughly 3.3 billion people are at risk for infection. This results in roughly 600,000 deaths, 90% of which are in Africa- of these the majority are children under the age of five (World Health Organization, World Malaria Report, 2014) (Bell et al., 2006). Disappointingly, this infection is both preventable and treatable. Although there has been a significant decline in transmission since 2000 due to insecticidal sprays, insecticide treated bed nets, rapid diagnostic tests as well as increased access to therapy and preventative therapy to pregnant women, there is also significant amount of work remaining with the increasing challenge of parasites resistant to current drugs and mosquitos resistant to insecticides.

Malaria in humans is caused by five parasite species, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and Plasmodium knowlesi.(Bell et al., 2006) This obligate intracellular parasite is transmitted by the bite of an anopheles sp. mosquito; a single successfully transmitted sporozoite can result in roughly 10,000 blood stage parasites, see schematic of parasite life cycle in Figure 1 below borrowed from a review (Lycett and Kafatos, 2002).

Figure 1.1] Parasite life cycle borrowed from (Lycett and Kafatos, 2002)
Progression of the parasite out of the liver stage, initiates the blood stage of infection which is associated with a majority of symptoms. The early symptoms are nonspecific and include headache, lassitude, fatigue, abdominal discomfort, and muscle and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise (Reyburn, 2010). Treatment of a patient at this stage results in full rapid recovery, thereby underscoring the importance of early detection and timely intervention. Treatment at a later stage, once organ dysfunction has ensued and parasite burden has increased, often results in rapid progression to severe malaria, particularly in the setting of a *P. falciparum* infection resulting in “coma (cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure or acute pulmonary oedema” (Reyburn, 2010). Severe malaria is almost always fatal when left untreated, and about 20% fatal even with treatment (Reyburn, 2010).

**Cerebral Malaria**

Cerebral malaria is the most severe manifestation of malaria infection and results in roughly 300,000 deaths annually. About 25% of survivors have lasting neurological complications (Christensen and Eslick, 2015; Fernando et al., 2010). In a group of Ugandan children the sequelae of cerebral malaria included spastic motor weakness, loss of speech, hearing deficits, behavioral problems, epilepsy, blindness, and severe cognitive impairment (Idro et al., 2010). Extensive neurological damage has been evidenced in pathology from patients who died from cerebral malaria. Demyelination, damaged neurons, (Schluesener et al., 1998; Torro and Roman, 1978) parasitized capillaries, petechial hemorrhages are among the many changes observed on autopsy specimens along with malarial retinopathy (hemorrhages, whitening and vascular changes) (Taylor et al., 2004). Due to the variability and complexity of disease presentation, pathologists have defined three predominant form of cerebral malaria defined by pathology “CM1—clinical cerebral malaria with sequestration of parasitized red blood cells (PRBCs) in the brain, no additional cerebral histopathological changes, and no other cause of death CM2—clinical cerebral malaria with sequestration of PRBCs in the brain and the presence of cerebral microthrombi, ring hemorrhages and extra-erythrocytic malaria pigment, and no other cause of death CM3—fulfilling the traditional definition of clinical cerebral malaria in life, but with no sequestration of PRBCs in the brain and another cause of death identified” (Dorovini-Zis et al., 2011; Milner et al., 2014; Taylor et al., 2004).

There are many models rationalizing the complex pathology present in cerebral malaria; the two prevailing hypotheses are 1.) sequestration/mechanical and 2.) immunopathology. The mechanical notion suggests that multifocal lesions result from reduced blood flow from sequestered parasites. This then causes metabolic changes: acidosis, hypoglycaemia, and hypoxemia which may conclude in a coma. However, this alone cannot account for the pathology since similar levels of sequestration have been seen in patients not suffering from cerebral malaria. The other dominant view suggests that an overactive immune response to the parasite results in endothelial damage and dysfunction which eventually results in a breakdown of the blood brain barrier and damage to the central nervous system as a byproduct of the immune system attempting to clear the parasite. Cerebral malaria is a complex pathological process and most likely results from a culmination of many factors.

**Mouse Models for Cerebral Malaria**

*Plasmodium berghei ANKA* infection of C57BL/6 mice is the most widely used murine model for cerebral malaria infection (mCM). This model replicates many of the clinical and histopathological features present in patients with human cerebral malaria (hCM). For example, mCM displays tissue edema, hemorrhages, presence of an inflammatory infiltrate, activation of microglia and a robust pro-inflammatory cytokine response. However, there are limited infecteded blood cells sequestered along the vascular endothelium and an a more robust inflammatory infiltrate than that present with hCM.

A very extensive debate on the validity of the model has been ensuing through articles and at conferences (Hunt et al., 2010; Renia et al., 2010; Riley et al., 2010; Stevenson et al., 2010; White et al., 2010). Amongst the most compelling arguments against using the murine cerebral malaria model is that 44 out of 48 therapies assessed in mCM model were successful in healing mice; however, of 17 assessed in human, only one has shown some efficacy. This underscores that the biology in these two distally related mammals may not be close enough to develop effective therapeutics.
The main features differentiating murine cerebral malaria and hCM are highlighted below in a table borrowed from a recent review (Medana et al., 2001).

<table>
<thead>
<tr>
<th>Observation</th>
<th>Human</th>
<th>Murine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of vascular cell integrity/tissue edema</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Congestion of microvessels with infected erythrocytes</td>
<td>+</td>
<td>-/+</td>
</tr>
<tr>
<td>Haemorrhages</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mononuclear cell adherence to, or extravasation through, the vascular endothelium</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>Astrocyte response (redistribution, astrogliosis, activation, apoptosis)</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Microglia and perivascular macrophage response (redistribution, morphological changes, activation)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pro-inflammatory cytokine expression</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neurological complications, including convulsions, paralysus, coma</td>
<td>+</td>
<td>+</td>
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Table 1] Major differences between human cerebral malaria and the murine model, *P. berghei* ANKA

Despite the controversy present around the *berghei* mouse model, there is some advantage to studies that use it. At a first pass, it is important to remember that the *berghei* model is at the end of the day a model and therefore an approximation of what happens in the human cerebral malaria case and not a replicate. It provides a glance to how different factors will affect the infection in the presence of an immune system, other organs, a vasculature, and many other components. It is of particular ease of use when studying factors exclusive to *P. falciparum* and not present in *P. berghei*. In this setting, the *berghei* model provides a background akin to a knock-out. As a consequence it is simple to compare infection in an isogenic background with one protein different. The studies have performed using *P. berghei* ANKA added a protein present only in *P. falciparum*, HRPII. We were able to demonstrate that addition of HRPII into the *berghei* genome reduced mouse survival and was therefore important for the parasite outside of simple parasite growth as suspected.

**HRPII**

Histidine rich protein II (HRPII) is a protein produced exclusively by *Plasmodium falciparum* and exported out of the parasitophorous vacuole into the red blood cell. Upon RBC lysis HRPII is released into the blood stream where it is found at concentrations > 1000ng/mL (Dondorp et al., 2005) and can be detected at least 1 month post clearance of parasites. HRPII is a curious protein not only due to its biochemical properties, but because it is only present in the *P. falciparum* genome. HRPII is highly basic, composed 37% by histidines; two repeats (His-His-Ala-His-Ala-Ala-Asp-Ala and His-His-Ala-Ala-Asp) cover 85% of its sequence. The sequence shown below highlights the histidine rich nature of the sequence. Since its discovery in 1986 (Wellems and Howard, 1986), many functions have been ascribed to it including hemozoin crystallization, actin formation, T cell suppression, glycosaminoglycan binding and procoagulation (Benedetti et al., 2003; Choi et al., 1999; Das et al., 2006; Mashima et al., 2002; Ndonwi et al., 2011; Sullivan et al., 1996). It has been shown to interact selectively with heparin, heparan sulfate and dermatan sulfate with high affinity (Ndonwi et al., 2011). This particular interaction led to the discovery of its procoagulant property, and may explain the pro-coagulant state seen during *P. falciparum* infection. Heparin at low concentrations is known to bind to the serpin antithrombin III and increase heparin’s inhibition of Factor Xa or thrombin 2000-4000 fold. HRPII has been shown by our lab to neutralize this enhancement of antithrombin activity and thereby promote a pro-coagulative environment. (Ndonwi et al., 2011)

Figure 1.2] Amino Acid sequence of HRPII from *P. falciparum*
HRPII is produced by almost all natural isolates of *Plasmodium falciparum*. Due to its presence at high concentrations in infected individuals it has been used as a biomarker for infected individuals and forms the basis of the dipstick test (Chiodini et al., 2007; Dondorp et al., 2005; Moody, 2002; Parra et al., 1991). The asexual stages of infection by all other species occur exclusively in the blood stream and therefore blood parasitemia is a decent indicator of parasite burden. In contrast, *P. falciparum* parasites in the trophozoite and schizont stages are sequestered along endothelial walls and can rarely be seen in the blood stream; therefore blood parasitemia is a poor indicator of parasite burden. Since HRPII is released into the blood stream as schizonts rupture, and is cleared from the blood stream slowly; HRPII serves as a good measure of recent *P. falciparum* infection.

HRPII has been used as a biomarker for *P. falciparum* infection and forms the basis of current rapid diagnostic tests (Chiodini et al., 2007; Dondorp et al., 2005; Moody, 2002; Parra et al., 1991). On post-mortem analyses, HRPII has been observed to line the endothelial walls of blood vessels (Aikawa et al., 1990). Several correlative studies have shown an association between HRPII levels in acute serum and disease severity or development of CM (Dondorp et al., 2005; Fox et al., 2013; Hendriksen et al., 2012; Hendriksen et al., 2013; Kariuki et al., 2014; Seydel et al., 2012). Natural populations of HRPII-deficient *Plasmodium falciparum* parasites exist (Gamboa et al., 2010; Koita et al., 2012; Kumar et al., 2013), though these tend to be in areas of low CM incidence. We questioned whether HRPII might contribute to disease pathogenesis.

**Blood Brain Barrier and Vascular endothelium**

The BBB regulates access of peripheral circulatory compounds and cells to the central nervous system. The BBB is formed by a complex network of intercellular junctional proteins. It is supported by many components a basement membranes, a complex extracellular matrix and various cells including astrocytes and microglia (Kawai and Akira, 2010). “Astrocytes, pericytes and extracellular matrix (ECM) components provide both structural and functional support to the BBB. The term ‘neurovascular unit’ (NVU) additionally refers to neurons, microglial cells and, optionally, peripheral immune cells that also contribute to this cellular interplay” (Obermeier et al., 2013). A schematic from a recent review is shown below Disruption of this network results in BBB compromise and has been linked to a various disease states.

**Fig 1.3: Neurovascular unit of the blood brain barrier. Figure borrowed from (Obermeier et al., 2013)**

The endothelial cells that line the vasculature in the brain are distinct from those in other organs in part due to the stringency and selectivity guiding which compounds can permeate past this endothelial lining. This selective barrier between the peripheral circulatory components of the blood and the brain, the blood brain barrier proper “lies in the presence of tight junctions between the cerebral endothelial cells of the vasculature of the brain both within the parenchyma and over the surface in the pia-arachnoid” (Stolp et al., 2013). Tight junctions prevent the paracellular transport of molecules into the parenchyma of the brain.
We used an in vitro model blood brain barrier to study how different components affect it. An illustration is shown below in figure 1.4. We culture a human brain cerebrovascular immortalized endothelial cell line hCMEC/D3 cells on collagen coated transwell inserts. The cells are allowed to grow to complete confluence and during this time they form appropriate tight junctions as previously described (Daniels et al., 2013; Weksler et al., 2005). We can measure the electrical resistance across this endothelial monolayer, high resistance values are indicative of an intact barrier while decreasing resistance values indicate a compromised model blood brain barrier.

**Figure 1.4] Model BBB**

**Inflammasome**

The innate and adaptive immune system work together to alert the host of danger signals both foreign and inappropriate host responses. The innate system is activated first and responds to a wide range of pathogenic or host patterns that have evolutionarily been deemed dangerous, and activates the adaptive immune response which is able to mount a specific response via B and T cells. (Basset et al., 2003). The innate immune system operates through PRRs (pathogen recognition receptors) which recognize PAMPs (pathogen associated molecular patterns). The innate immune system can also be activated by components released by injured cells termed danger associated molecular patterns (DAMPs) such as mammalian double stranded DNA and uric acid crystals (Ishii et al., 2001; Martinon et al., 2006). So the innate immune system recognizes common patterns from invading bacteria viruses and fungi as well as damage resulting from the invasion to the host. PRRs can be cytoplasmic, membrane bound or even secreted and are present in specialized immune cells such as macrophages, monocytes, dendritic cells (DCs), neutrophils, in addition to normal mononuclear endothelial and epithelial cells. Toll-like receptors (TLRs) are one set of well-established PRRs (O'Neill and Bowie, 2007). More recently discovered PRRs are RIG-like helicases (RLH) and NOD- like receptors (NLRs) which are soluble cytoplasmic receptors unlike TLRs which are membrane bound (Martinon and Tschopp, 2005; Yoneyama et al., 2004). NLRs are a common class of sensor molecules for inflammasomes. Inflammasomes are large molecular weight complexes that recognize pathogenic or sterile danger molecules and activate the pro-inflammatory cytokines IL-1β (interleukin 1 beta) and IL-18 (interleukin 18) (Latz et al., 2013). The complex is formed from the association of a sensor molecule, the adaptor protein ASC which recruits caspase-1 and caspase-1. The adaptor protein ASC has two death- fold domains: a pyrin domain and a caspase activation and recruitment domain (CARD) (Vajjhala et al., 2012). The pyrin domain allows for association with the upstream sensor molecule and the CARD domain brings pro caspase-1 molecules in close proximity allowing cleavage and self-activation. Active caspase-1 molecules are able to cleave pro- IL-1β and pro IL-18 into their respective active forms IL- 1β and IL-18. Many sensor molecules have been identified such as NLRP1, NLRP3, NLRP12, NAIP1, NAIP2, NAIP5, or AIM2 and are collectively able to detect a diverse array of host and pathogenic danger signals. Activation of the
inflammasome eventually results in a form of programmed inflammatory cell death known as pyroptosis. Although activation of the inflammasome is often thought of in the context of a bacterial infection or host danger molecules, it can also be activated from an intracellular protozoan parasite infection (Zamboni and Lima-Junior, 2015).

The work that follows was pursued to understand the function of HRPII, a parasite protein that is biochemically very unique, produced at high levels and whose serum levels correlate with disease severity and cerebral malaria. We reasoned that a function should exist since the protein is produced to such high levels. In addition, early work had shown that parasites were viable in the presence of a genetic deletion of HRPII, in fact, natural HRPII null parasites exist. Therefore, it hypothesized that a function for HRPII, if existed, would be not be in parasite growth, but perhaps something important during the life cycle in the host. In consideration of the correlation between HRPII levels and cerebral malaria, future study HRPII on human brain endothelial cells.

Fig 1.5 Inflammasome highlighting the many points of activation borrowed from (Latz et al., 2013)

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References


