Looking through a cranial window
Intravital microscopy for in vivo study of cerebral malaria

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Malaria is a potentially life-threatening disease with 200–500 million new infections and up to one million deaths annually.1 Half the world’s population is at risk of being infected by one of the five Plasmodium parasite species causing malaria in humans. P. falciparum is responsible for the most fatal outcomes occurring in sub-Saharan Africa.2 Infection can progress very fast from a mild into a severe form featuring typical symptoms such as anemia, respiratory abnormalities, cerebral malaria (CM), and coma.3 Children under the age of five are most susceptible to the development of CM and account for most deaths.4,5 Patients who survive CM are likely to suffer long-standing neurocognitive impairment.6-8 The underlying pathogenesis of CM is not well understood. A histological hallmark of CM is the sequestration of the parasite-infected red blood cells to the microvasculature of the brain.9,10 Multiple factors such as microvascular obstruction, vascular inflammation, hemostasis dysfunction, reduced microcirculatory blood flow, and breakdown of the blood–brain barrier (BBB) are all considered to be important contributors to the development of CM.11-13 In order to develop successful adjunctive therapy to anti-malarial treatment to prevent neurological impairment in patients, an improved understanding of CM pathogenesis is essential.

The study of CM in humans is restricted to the correlation of different pathologies to define and diagnose CM and to the examination of postmortem samples,14-16 which reveal insight into histological patterns associated with the disease. The exact mechanisms leading to human CM are largely unknown and previous therapeutic attempts were based on the observed pathophysiology in humans.17,18 For this reason, murine malaria models such as the Plasmodium berghei ANKA (PbA) model have been introduced to gain a better understanding of the mechanisms contributing to the development and protection of experimental cerebral malaria (ECM). While this model does not reflect the complexity of disease observed in humans, similarities in CM pathogenesis such as breakdown of the BBB and vascular damage make it to date the best available small animal model for severe malaria and ECM.19-21 A major concern regarding the suitability of the ECM model has been based on observations that sequestration to endothelial cells of the brain during human CM is caused by P. falciparum infected RBCs, while in the ECM-susceptible C57BL/6 mouse strain infected with PbA leukocytes appear to take this role.22 Important in this context, only recently has the first direct in vitro evidence been provided demonstrating sequestration of PbA infected RBCs to endothelial cells of mouse brain involving host receptor VCAM-1 as a potential mediator of adherence.23

One way to gain important information regarding underlying pathological features of the microvasculature contributing to ECM progression has been made feasible through recent implementation of intravital microscopy through a closed cranial window by Carvalho and colleagues.24,25 This tool allows for the first time continuous in vivo analysis of the pial microcirculation in a single area of the brain, which reflects general brain circulation behavior and function including BBB properties.26 Importantly, pathological changes during ECM progression in the pial vessels (pial arterioles and pial venules) appear similar to the ones observed in brain parenchym vessels. This includes hemodynamic and inflammatory changes due to leukocyte sequestration, vascular occlusion, hypoperfusion, leakage, and microhemorrhages.22

In previous studies, intravital microscopy revealed that vasooconstriction is caused by decreased cerebral blood flow and is closely associated with ECM.22 A correlation of the number of sequestering leukocytes to the vessel endothelium and the severity of vasoconstriction was observed, causing decreased blood flow and cellular hypoxia, and potentially leading to vascular collapse. With the recognition that vasolopathy appears to be an important contributor to the pathogenesis of CM, the successful treatment of ECM with the calcium-channel blocker and vasodilator nimodipine combined with antimalarials was performed and monitored by intravitral microscopy.22 In another study, intravital microscopy was applied to demonstrate that supplementation of nitric oxide supported prevention of vasoconstriction, leukocyte sequestration, and development of brain hemorrhages.27 This finding pointed to a role of nitric oxide synthases in cerebrovascular dysfunctions during ECM and complemented previous observations that treatment of patients and mice with exogenous arginine increased nitric oxide production and resulted in reduced BBB perfusion during progression of CM.25,30

Vascular dysfunction is a major feature of human CM and alterations of cerebral microcirculation lead to cerebral hypoxia.33,31

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Whitening of the retina, hemorrhage, and changes in vessel color due to parasite sequestration and obstruction of blood flow have been interpreted as indirect indications of reduced cerebral oxygenation linked to CM. Since sufficient brain oxygenation is vital to resolve clinical consequences of a *P. falciparum* infection, a better understanding of cerebral hypoxia associated with CM is essential for the development of novel interventions.

The article in this issue of *Virulence* by Cabrales et al. describes for the first time a direct, quantitative, and dynamic in vivo measurement of hypoxia during ECM development using intravital microscopy through a closed cranial window. The technique is combined with phosphorescence-quenching microscopy to measure oxygen tensions and pH-sensitive fluorescence to assess pH values of perivascular tissue. Individual vessels of ECM-susceptible C57BL/6 mice and ECM-resistant BALB/c mice infected with *PbA* were investigated for blood flow, oxygen tension, and transport. With the onset of ECM development, decreased pH values and drop of the core body temperature in C57BL/6 mice were observed, potentially affecting cell function and blood flow. PbA infection in the ECM-susceptible mice induced compromised oxygenation and impaired pial hemodynamics, while ECM-resistant mice had better oxygen delivery and extraction levels. Reduced oxygen delivery was correlated with the number of sequestering leukocytes during early and late ECM. Resulting vascular obstruction and vasoconstriction promoted anaemia and hypoperfusion, which further impaired oxygenation. This led to increased acidosis, hypothermia with potential neurological damage developing.

In summary, Cabrales et al. present for the first time a quantitative analysis of fluctuations of oxygen transport and tension during ECM progression and its contribution to the severity of disease. The study highlights the pial tissue as highly sensitive to changes of blood flow, anaemia, and low oxygen tension impacting sufficient oxygen delivery. Further, the findings herein complement previous observations of changes in energy metabolism and transcription factors marking the state of hypoxia.

It becomes apparent that the use of intravital microscopy carries a huge potential for the mechanical dissection of microvascular changes as part of the underlying pathogenesis of CM. It also facilitates the development of new therapeutics for the prevention of CM by allowing long-term imaging with testing effects of new compounds on the microvasculature directly after administration. Intravital microscopy may also be applied for the analysis of the contribution of genes to the development of vasculopathy, which may be performed through gene deletion approaches. Cytoadherence to the cerebral endothelium mediated by infected RBCs and leukocytes may be studied by intravital microscopy, leading to identification of underlying mechanisms determining different vascular conditions during the progression of CM.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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