Cytoadherence of *Plasmodium berghei*-Infected Red Blood Cells to Murine Brain and Lung Microvascular Endothelial Cells *In Vitro*

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Sequestration of infected red blood cells (iRBC) within the cerebral and pulmonary microvasculature is a hallmark of human cerebral malaria (hCM). The interaction between iRBC and the endothelium in hCM has been studied extensively and is linked to the severity of malaria. Experimental CM (eCM) caused by *Plasmodium berghei* ANKA reproduces most features of hCM, although the sequestration of RBC infected by *P. berghei* ANKA (PbA-iRBC) has not been completely delineated. The role of PbA-iRBC sequestration in the severity of eCM is not well characterized. Using static and flow cytoadherence assays, we provide the first direct *in vitro* evidence for the binding of PbA-iRBC to murine brain and lung microvascular endothelial cells (MVEC). We found that basal PbA-iRBC cytoadherence to MVECs was significantly higher than that of normal red blood cells (NRBC) and of RBC infected with *P. berghei* K173 (PbK173-iRBC), a strain that causes noncerebral malaria (NCM). MVEC prestimulation with tumor necrosis factor (TNF) failed to promote any further significant increase in mixed-stage iRBC adherence. Interestingly, enrichment of the blood for mature parasites significantly increased PbA-iRBC binding to the MVECs prestimulated with TNF, while blockade of VCAM-1 reduced this adhesion. Our study provides evidence for the firm, flow-resistant binding to endothelial cells of iRBC from strain ANKA-infected mice, which develop CM, and for less binding of iRBC from strain K173-infected mice, which develop NCM. An understanding of *P. berghei* cytoadherence may help elucidate the importance of sequestration in the development of CM and aid the development of antibinding therapies to help reduce the burden of this syndrome.

First described in the 19th century, sequestration, the cytoadhesion of infected red blood cells (iRBC) within the microvasculature, is a common feature of human cerebral malaria (hCM) (1–3). Cerebral malaria (CM) is a life-threatening encephalopathy, a complication of *Plasmodium falciparum* infection. In the human host, it is characterized by coma and is quantitatively associated with cerebral sequestration and total parasite biomass (4–6). The progression of uncomplicated malaria to potentially fatal CM remains poorly understood, though it is associated with microcirculatory dysfunction due to the formation of cerebral lesions (4, 7, 8). These lesions develop when mature forms of the parasite, trophozoites, or schizonts or immature gametocytes bind to the endothelial cells (EC) lining small capillaries and post-capillary venules and consequently plug the vessel lumen, placing pressure on tight junctions of the blood-brain barrier (BBB) (9, 10). This causes downstream hypoxia within the tissue, resulting in poor perfusion and damage to adjacent tissues (11). This, coupled with the dysregulated release of cytokines and chemokines from immune cells, contributes to the development of CM (7, 11, 12).

The vaso-occlusive sequestration of iRBC in vital organs has potent effects on organ function. The majority of sequestered parasites are found in the brain, lung, spleen, liver, kidney, small intestine, heart, and adipose tissue, as well as in the placenta in pregnancy-associated malaria (3, 13, 14). The microaerophilic venous environment of the deep microvessels nourishes the maturation of the parasites, facilitating their survival (15).

The pathogenicity of *P. falciparum* is partially attributed to its ability to evade the immune system, particularly the filtration by the spleen, through adherence to the vascular endothelium (16–18). Numerous studies have directed their efforts at identifying parasite virulence ligands and host endothelial cell receptors and adhesins that mediate this cytoadhesion, with the goal of developing strategies that will reduce the incidence of CM (7, 19, 20). *P. falciparum* sequestration is mediated by the interactions between parasite ligand *P. falciparum* EMP1 (PiEMP1), displayed on the surface of iRBC, and multiple receptors, such as ICAM1, CD36, CD31, and CSA (20–23), displayed on the surface of the host cell.

Murine models of CM, particularly *P. berghei* ANKA, are well established in the study of CM pathogenesis (24) and are utilized as a preclinical model for drug testing (25). The strain ANKA model of experimental CM (eCM) replicates most signs of hCM (12, 26, 27), although eCM is mainly characterized by monocyt/macrophage, T cell, and platelet sequestration (28–34) as opposed to iRBC sequestration, which is more prominent in hCM (35, 36). Some argue that this binding is not mediated by the interaction of parasite ligands and endothelial receptors but rather an accumulation or trapping in small blood capillaries that is more representative of stasis. For this reason, attempts have been made at creating mouse models that closely resemble hCM and reproduce the signature iRBC microvascular sequestration (34). The field is con-
The interaction between iRBC and the endothelium is not passive. Parasite proteins interact with the host RBC to change its morphology, physiology, and function (43). Parasites produce mediators that trigger the release of cytokines from a range of host cells, including EC. These cytokines facilitate cytoadherence by upregulating the expression of ligands available on the surface of host cells, and this interaction activates signaling cascades that downregulate genes involved in the inflammatory response and apoptosis (44). In eCM, tumor necrosis factor (TNF) has long been known to be an important mediator in the pathogenesis of CM (45–47). A number of factors support the adhesion of the parasite to the endothelium, including host cells such as macrophages (47), lymphocytes (48), platelets (49), and plasma microparticles (50). IRBC can directly stimulate the endothelium in the absence of proinflammatory cytokines, inducing the expression of adhesion molecules and chemokines and mimicking the effects caused by cytokines such as TNF (51–55).

Sequestration of IRBC in the microvasculature has been implicated in the severity of *P. falciparum* infection, as reviewed in reference 56, and in eCM, with *P. berghei* ANKA sequestering in a more controversial, and of the few studies on strain ANKA binding in vivo (33, 34, 37–41), one suggests that sequestration is not linked to the development of CM (33), and this has challenged the relevance of the murine model to the human syndrome (42).

more, we assessed the involvement of the endothelial molecules ICAM-1 and VCAM-1, known to mediate *P. falciparum* cytoadherence and present on our MVEC lines, in the observed binding.

**MATERIALS AND METHODS**

Research procedures were approved by the Animal Ethics Committee of the University of Sydney (K20/7–2006/3/4434 and K80/10–2010/3/5317).

**Microvascular endothelial cells.** Primary mouse brain MVEC (B3) were isolated as described previously (63). Primary mouse lung MVEC (L2) were derived from primary cultures of peripheral lung parenchyma of CBA/Ca mice. These cells have been immortalized by transfection of a plasmid construct containing Bcl-xl protein. Cells were seeded in culture flasks and grown to confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO₂.

**Surface phenotyping of B3 and L2 cells.** Monolayers of B3 and L2 cells were cultured until confluence and then either left unstimulated or incubated with recombinant murine TNF (10 ng/ml; Peprotech) for 4 h or overnight. Cells were then washed in phosphate-buffered saline (PBS) and exposed to a short trypsin-EDTA treatment to harvest a cell suspension. To identify resting and activated cellular markers, the cells were directly labeled using anti-mouse monoclonal antibodies directed against PECAM-1, ICAM-1, integrin beta-3, endoglin, VCAM-1, and E-selectin (CD31, CD54, CD61, CD105, CD106, and CD62E, respectively; Biocytex) and their respective isotype-matched immunoglobulins. Cells were then resuspended in PBS prior to flow-cytometric analysis (Beckman Coulter FC500). E-selectin was measured after a 4-h TNF treatment, while the remaining molecules were measured after an overnight treatment.

**Preparation of red blood cells.** Infection was induced in 8-week-old CBA female mice by intraperitoneal injection of 1 × 10⁷ *P. berghei* ANKA- or 2 × 10⁷ *P. berghei* K173-parasitized RBCs as described previously (26, 27). Blood was collected in citrated tubes via retro-orbital sampling from infected mice 6 days postinfection, while noninfected mice were used as controls. Parasitemia was determined by Diff-Quick-stained thin tail vein blood smears.

Red blood cells were separated from whole blood by centrifugation at 500 × g for 5 min and were washed at low speed in PBS three times. Mixed-stage RBC were used at 6 to 7% parasitemia. Mature-stage iRBC were selected and concentrated using automatic magnetically activated cell sorting (MACS) as previously described (64). Enriched RBC were used at 40% parasitemia with 70% of the parasites at the mature stage. *P. falciparum* Pb-fam-1, and *P. berghei* ANKA sequestering in *P. falciparum* infection, as reviewed in reference 56, and in eCM, with *P. berghei* ANKA sequestering in a more controversial, and of the few studies on strain ANKA binding in vivo (33, 34, 37–41), one suggests that sequestration is not linked to the development of CM (33), and this has challenged the relevance of the murine model to the human syndrome (42).

**Static cytoadherence assays.** B3 and L2 cells were cultured until confluence on 24-well plates. Cells were either left unstimulated or were activated overnight with 50 ng/ml murine TNF. The MVEC were then washed in medium and incubated with RBC (RBC/MVEC ratio, 20:1) for 90 min at 37°C. Nonadherent RBCs were then removed by gentle washing in medium, followed by more thorough washing with PBS and plate inactivation. Remaining cells were fixed by incubation with 1% (wt/vol) paraformaldehyde at 4°C overnight. Bound RBCs were counted on at least 15 fields from 3 separate experiments imaged on an Olympus IX71 inverted microscope (×400 magnification).

**Flow cytoadherence assays.** Cell adherence assays were performed using parallel-plate flow chambers (Glycotech). Confluent monolayers of B3 and L2 cells, seeded in 35-mm petri dishes, were stimulated with TNF by following the same regimen as that for the static conditions. RBC were resuspended at 1% hematocrit and perfused over endothelial monolayers for 15 min at a shear stress of 0.5 dynes/cm². Plates were washed and fixed prior to imaging (Olympus IX71 inverted microscope at ×400 magnification).
RESULTS

Adhesion of *P. berghei*-iRBC to murine brain and lung microvascular endothelial cells under static conditions. Static assays were performed as described in Materials and Methods; notably, mixed-stage iRBC were incubated for 90 min on resting or activated (TNF, 50 ng/ml) endothelial cells. More PbA-iRBC than NRBC and PbK173-iRBC bound to both B3 and L2 MVECs. Data shown are the mean numbers ± SEM of RBC per field of view from at least 15 fields from 3 separate experiments (*, P < 0.05).

Firm, flow-resistant binding of PbA-iRBC to murine brain and lung microvascular endothelial cells. RBC were resuspended at 1% hematocrit and perfused over endothelial monolayers for 15 min at a flow rate of 0.227 ml/min in a flow chamber. More PbA-iRBC adhered than did NRBC, despite a shear stress of 0.05 Pa being maintained in the flow chamber. Data shown are the mean numbers ± SEM of RBC per field of view from at least 15 fields from 3 separate experiments (**, P < 0.05).

The upregulation of adhesion molecules ICAM-1 and VCAM-1 on the surface of B3 and L2 cells represents potential receptors involved in the adhesion of mature PbA-iRBC. Following overnight TNF stimulation, both ICAM-1 and VCAM-1 were markedly upregulated on B3 and L2 cells (MFI, 4.54 versus 10.5; 5.55 versus 20).

Enrichment for mature parasites increases the level of PbA-iRBC binding on MVECs prestimulated with TNF, and blocking VCAM-1 reduces this binding. To investigate the effect of TNF on the binding of iRBC harboring mature stages of the parasite, blood was enriched for mature forms and incubated on B3 monolayers in resting and activated conditions. Prestimulation with TNF significantly increased the number of mature PbA-iRBC bound compared to that of NRBC (Fig. 2). As seen in the static assays, MVEC prestimulation with TNF failed to induce any further increase in RBC adherence, and fewer RBC bound to lung MVEC monolayers than brain MVEC monolayers.

FIG 2 Firm, flow-resistant binding of PbA-iRBC to murine brain and lung microvascular endothelial cells. RBC were resuspended at 1% hematocrit and perfused over endothelial monolayers for 15 min at a flow rate of 0.227 ml/min in a flow chamber. More PbA-iRBC adhered than did NRBC, despite a shear stress of 0.05 Pa being maintained in the flow chamber. Data shown are the mean numbers ± SEM of RBC per field of view from at least 15 fields from 3 separate experiments (**, P < 0.05).

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receptors by which the RBC were binding under experimental inflammatory conditions (Fig. 4). To address this possible interaction, B3 monolayers were incubated with purified anti-mouse ICAM-1 or anti-mouse VCAM-1 (LEAF) and their respective isotype-matched controls. These monoclonal antibodies selectively block ICAM-1 binding to LFA-1 and Mac-1 receptors, as well as VCAM-1 binding to VLA-4 (α4β1 integrin) and LPAM-1 (α4β7 integrin) receptors. A 65% reduction in the number of PbA-iRBC was observed when blocking VCAM-1, whereas no effect was seen when blocking ICAM-1 or when the isotype-matched controls were used. The addition of the blocking antibodies had no effect on the number of binding NRBC compared to that of isotype controls.

**DISCUSSION**

This study describes our findings on the cytoadherence of *P. berghei*-iRBC to murine brain (B3 cells) and lung (L2 cells) MVEC. First, our data provide the first direct *in vitro* evidence, to our knowledge, that PbA-iRBC are capable of cytoadhering to murine brain and lung MVEC. Second, the proportion of binding is higher for RBC infected with strain ANKA, the strain causing eCM, than those infected with strain K173, the strain causing NCM, and this binding is independent of endothelial cell pre-stimulation by TNF. Third, this binding is firm, shear stress resistant, and significantly higher on brain than on lung MVEC. Finally, enriched mature PbA-iRBC bind to TNF-activated MVEC to a greater extent than to resting MVEC, and this binding is reduced when VCAM-1 is blocked on the endothelial surface.

In our study, the binding of fluorescent iRBC from strain ANKA- and strain K173-infected mice to brain MVEC was clearly visible, with PbA-iRBC binding more than PbK173-iRBC. Brain sections from mice infected with the nonsequestering line *P. berghei* K173 display little to no sequestered parasites (69), similar to what is observed in severe human malaria without coma (3, 4, 6, 14, 36, 70, 71). On lung MVEC we also observed binding of PbA-
iRBC, although overall it was lower than that observed on brain MVEC. We found no difference in binding of mixed-stage PbA-iRBC in resting or TNF-activated conditions (Fig. 1). The cytoadherence-linked virulence of the parasite, as displayed by strain ANKA, may support its ability to influence the development of CM in the host. It would be advantageous for early-stage parasites to directly activate the host endothelium to support adhesion in order to enable survival and transmission in vivo (51). In our system, RBC were sampled directly from the peripheral blood of mice on the day of experimentation; thus, there is no influence of long-term culture, i.e., loss of the cytoadherent phenotype (72).

Based on these observations, a series of flow assays was performed to confirm the binding under physiological conditions, similar to what is observed inside a microvessel. PbA-iRBC binding withstood flow conditions, confirming that the majority of the interactions did not resemble stasis or weak binding but true cytoadherence, and the interaction was firm and flow resistant (Fig. 2). However, we did observe lower levels of binding under flow conditions across the brain and lung endothelium, confirming that shear stress rates significantly affect cytoadherence, and that some of the binding seen under static conditions is indeed due to stasis or weak binding.

Our data demonstrate that increased binding of mature parasites compared to mixed-stage parasites occurs following TNF stimulation. In vivo, strain ANKA schizonts disappear from the peripheral circulation and have a distinct sequestration phenotype (33, 73, 74). In our system, we hypothesized that mature parasites and schizonts would bind more than mixed-stage parasites, as seen with P. falciparum (3, 75). Surprisingly, under resting conditions these mature parasites bound no differently than NRBC. However, under inflammatory conditions (TNF-stimulated monolayers), we observed a significant increase in the number of bound mature-stage PbA-iRBC (Fig. 4).

In order to determine the possible binding mechanism present in our assay, we studied inducible receptors present on the surface of the MVEC. From the candidate receptors present on B3 and L2 cells (Fig. 3), we focused on those upregulated following TNF activation, namely, ICAM-1 and VCAM-1, mimicking the inflammatory conditions seen in CM. Previous studies have shown that ICAM-1 and VCAM-1 expression is upregulated by TNF on endothelial cells, and these molecules are receptors for P. falciparum (76). ICAM-1 becomes upregulated on the brain and lung endothelium during eCM (77–79) and is known to mediate iRBC binding to the vascular endothelium (80). Moreover, there is evidence for the colocalization of sequestration and ICAM-1 expression in the brain in CM (81). Surprisingly, in our study, inhibition of ICAM-1 had no significant effect on binding of mature-stage PbA-iRBC to brain (Fig. 4) or lung MVEC (data not shown). Further experiments using other clones of ICAM-1 are required to confirm whether this lack of effect is due to the blocking antibody or the noninvolvement of ICAM-1.

There is evidence for a role for VCAM-1 in P. falciparum sequestration (76, 82, 83). In clinical malaria, P. falciparum-infected RBC roll and adhere to VCAM-1, although the epitope involved in the interaction is not yet known (84, 85). Our study demonstrates that VCAM-1 may be important in maintaining the adherence of mature PbA-iRBC to the cerebral endothelium (Fig. 4), since binding was inhibited in the presence of an anti-VCAM-1 monoclonal antibody (Fig. 4). The binding of PbA-iRBC in cultures may be a result of the interaction between VCAM-1 and parasite ligands or VLA-4-like antigen present on the surface of RBC, although further studies are required to determine this.

In other studies, CD36 has been identified as the host cell receptor for PbA-iRBC adherence. This is also one of the major receptors of P. falciparum iRBC and could account for the differential binding observed on brain and lung MVEC (33). Orthologues of PfEMP1 are absent from P. berghei and, as yet, strain ANKA ligands for CD36 have not been identified (62). Interestingly, mice express little or no CD36 in the brain (86) and CD36-mediated sequestration of P. berghei is not necessary for eCM, since cerebral complications still develop in the absence of this molecule (33). Although the lung is a site of pathology in CM, pulmonary sequestration of iRBC is not necessary for the development of acute lung injury or CM (87). However, peripheral parasite burden induces acute lung injury in eCM (79). In mice infected with the nonencephalitic strain, K173, lung pathology develops in the absence of CM (88) and at low inoculum size can induce CM without evidence for sequestration (89, 90). Alternative adhesion pathways that may be responsible for the binding are still unknown.

Our work demonstrates that mixed-stage PbA-iRBC, containing the parasite strain that causes CM, can bind firmly to the cerebral endothelium in the absence of TNF. However, mature parasites may require TNF to bind, and VCAM-1 could be mediating this binding (Fig. 4). Our in vitro model isolates three major components of the cerebral lesion: the endothelial cell, the RBC, and the parasite. However, there are other effectors in the CM lesion; thus, our data require careful interpretation. Nevertheless, target organ-specific cell lines, such as B3 and L2, expressing cytoadherence receptors for PbA-iRBC may provide a solid in vitro system that supplements the eCM model to study molecular mechanisms of strain ANKA binding. Future work may involve the addition of platelets, MP, and leukocytes to the system in order to model closely what is seen in vivo (10, 49). Existing eCM models enable access to a large library of genetically modified mice from which MVEC from various organs could be purified. Further experiments are required to identify which receptors are recognized by P. berghei parasites and discern the specific parasite stages of the intraerythrocytic cycle that bind. Ultimately, this could reveal how parasites use cytoadherence to evade the immune system inside the host, how they contribute to the severity of disease, and how therapies work at interfering with this binding to lessen the global burden of CM (91, 92).

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F.E. designed and performed experiments, collected and analyzed data, and wrote the manuscript. J.W. performed phenotyping experiments and interpreted data. A.J.M and N.H.H. provided reagents, technical support, and conceptual advice and interpreted data. G.E.G and V.C. designed the study, gave conceptual advice and technical support, analyzed data, and edited the manuscript.

We declare no competing financial interests or conflicts of interest.

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