Platelets Potentiate Brain Endothelial Alterations Induced by *Plasmodium falciparum*

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Brain lesions of cerebral malaria (CM) are characterized by a sequestration of *Plasmodium falciparum*-parasitized red blood cells (PRBC) and platelets within brain microvessels, as well as by blood-brain barrier (BBB) disruption. In the present study, we evaluated the possibility that PRBC and platelets induce functional alterations in brain endothelium. In a human brain endothelial cell line, named HBEC-5i, exhibiting most of the features demanded for a pathophysiological study of BBB, tumor necrosis factor (TNF) or lymphotoxin α (LT-α) reduced transendothelial electrical resistance (TEER), enhanced the permeability to 70-kDa dextran, and increased the release of microparticles, a recently described indicator of disease severity in CM patients. In vitro cocultures showed that the presence of platelets or PRBC can have a direct cytotoxic effect on activated, but not on resting, HBEC-5i cells. Platelet binding was required, as platelet supernatant had no effect. Furthermore, platelets potentiated the cytotoxicity of PRBC for TNF- or LT-α-activated HBEC-5i cells when they were added prior to these cells on the endothelial monolayers. This effect was not observed when platelets were added after PRBC. Both permeability and TEER were strongly affected, and the apoptosis rate of HBEC-5i cells was dramatically increased. These findings provide insights into the mechanisms by which platelets can be deleterious to the brain endothelium during CM.

Malaria results in up to 2.5 million deaths annually, with young children and pregnant women at greatest risk. *Plasmodium falciparum* can cause the most severe and life-threatening form of malaria in humans: cerebral malaria (CM). A characteristic feature of *P. falciparum* infection is the sequestration of parasitized red blood cells (PRBC) in various organs, such as the brain, lung, and placenta. While the mechanism of PRBC accumulation has been studied extensively, its role in the pathogenesis of fatal disease remains poorly understood (4, 13, 26). Sequestration in postcapillary venules and capillaries results from the interaction between adhesive, parasite-derived molecules expressed on the surface of the infected red cells and one or more of several receptors expressed on the surface of the vascular endothelium (16, 58).

The expression of some of these adhesion molecules on the surface of endothelial cells (EC) is modulated by cytokines (5, 48), notably tumor necrosis factor (TNF). Apart from this involvement in CM pathogenesis (27, 37), TNF induces profound alterations in microvascular EC, including morphological reorganization (54, 55, 63), junction protein redistribution (9), and production of proinflammatory cytokines and apop-tosis (24, 33, 42). Disruption of the blood-brain barrier (BBB) structure and function during CM has been described. The distribution of the cell junction proteins occludin, vinculin, and ZO-1 was altered in patients with CM compared to controls (10). It has been suggested that these functional BBB changes could possibly result from the binding of PRBC to cerebral EC. More recently, lymphotoxin α (LT-α), previously called TNF-β, has been proved to play a crucial role in murine CM (21), but its importance in human pathogenesis has not been clearly established yet.

Apart from PRBC (61), leukocytes (monocytes and macrophages) have been reported to sequester in brain vessels in patients with CM (29, 51, 52, 56). Platelets also accumulate in the cerebral microvessels of patients who die from CM, and this accumulation was shown to be significantly higher in patients who died of CM than in those who died of other causes (29). More recently, platelets were shown to be involved in PRBC sequestration: in vitro, platelets can act as a bridge between PRBC and TNF-stimulated endothelium. They might therefore promote or decrease cytadherence throughout the microvasculature, by providing new parasite receptors or by hiding expressed receptors on the surface of EC (75).

In addition to their role in mechanical vessel obstruction, platelets and PRBC may have direct effects on endothelial integrity. It has been demonstrated that platelets act in vitro as effectors of vascular endothelium damage, after TNF stimulation of brain EC (11). So far, the effect of both platelets and PRBC on functional changes or viability of human brain EC (HBEC) has not been studied in a coculture model of CM. Moreover, human CM studies have been performed using only...
noncerebral macro- or microvascular EC such as human umbilical vein EC (17, 76), human lung EC (49, 67), human dermal microvascular EC (47, 77), and Saimiri monkey brain EC (58). Indeed, it has been shown that, due to tissue specificities, there is marked endothelial heterogeneity, related in some way to the function of the organ in which the cells are found (6, 44, 60).

The fact that (i) platelet accumulation was significantly higher in the microvascularization of Malawian children who died of CM than in brain microvessels of control patients (29), (ii) PRBC enhanced EC apoptosis in an in vitro model of CM (53), and (iii) platelets could act in vitro as effector cells of vascular damage on TFN-pretreated mouse brain EC (45) prompted us to improve a preexisting in vitro model of the CM lesion, involving human brain endothelium, platelets, and PRBC.

MATERIALS AND METHODS

Isolation, purification, and immortalization of HBEC-5i cell line. Primary human brain EC (HBEC-5i) were derived by Dorovini-Zis and colleagues from small fragments of human cerebral cortex obtained from patients who died of various causes (18). These brains were devoid of any pathological abnormalities, as described, and the isolation and purification procedures were carried out according to the method developed by Bowman and colleagues (7) with minor modifications (18). For transfection and subcultivation, HBEC-5i cells were cultured in medium 199 (GIBCO BRL, Gaitersburg, Maryland) with 15% (vol/vol) fetal bovine serum (HyClone, Logan, Utah) and supplemented with 0.146 mg/ml L-glutamine, 10 mM HEPES, 2.2 mg/ml sodium bicarbonate, 40 mg/ml endothelial cell growth supplement (Collaborative Biomedical Products), and 16 U/ml sodium heparin (Sigma Chemical Co., St. Louis, Mo.). Cells were incubated at 37°C in 5% CO2 in water-saturated air. Primary HBEC-5i cells in their fifth passage from isolation were transfected using a plasmid designated pSVT, to produce a stable and immortalized cell line (20, 74). This plasmid was a pBR322-based plasmid construct, containing sequences that coded for the large T transforming protein of simian virus 40 (SV40) and the Rous sarcoma virus long terminal repeat. Single-donor endothelium was plated onto six-well tissue culture dishes (Costar, Cambridge, Mass.) with a suspension of 3.5 × 104 cells per well. HBEC-5i cells were incubated overnight at 37°C, 5% CO2. Then 2, 5, or 10 μg of pSVT DNA was added per well. Twenty-four hours after the transfection procedure, cells in each well were transferred to individual 25-cm2 tissue culture flasks and observed for growth and morphology changes. After two subcultures, transfected HBEC-5i cells were analyzed for the expression of SV40 large T antigen by enzyme-linked immunosorbent assay, with a monoclonal anti-SV40 large T antigen antibody (MAB 101, 100 ng/ml) and then resuspended and washed in Tyrode buffer, and the suspension was centrifuged (13,000 × g, min). Pellets containing platelets and debris were discarded, and MP present in the supernatants were double labeled by annexin V-FITC–anti-CD54-phycoerythrin (FITC) and then resuspended in binding buffer (Beckman-Coulter Immunotech) as previously described (14).

(iii) Vesiculation quantitation. HBEC-5i cells were seeded and grown to confluence in culture flasks. Cells were then left unstimulated or activated by TNF (100 ng/ml) or LT-α (100 ng/ml) for 6 h for scanning electron microscopy and processed as described above for scanning microscopy. To measure the number of microparticles (MP) released, cells were stimulated with the same agonists overnight and cultured supernatants were collected and centrifuged at 1,500 × g for 15 min to discard dead EC and debris. Endothelial microparticles were labeled using annexin V-fluorescein isothiocyanate (FITC) and then resuspended in binding buffer (Beckman-Coulter Immunotech) and analyzed by flow cytometry. The percentage of MP positive for both annexin V and CD54 were then quantitated.

Platelets. Blood was collected from healthy volunteers into Vacutainer tubes containing 0.129 M buffered sodium citrate. The volunteers had not taken any drugs for at least 14 days. Platelets were isolated as previously described (75). Briefly, platelets were purified by centrifugation of a platelet-rich plasma sample and then resuspended and washed in Tyrode buffer, and the suspension was adjusted to 2 × 109 platelets/ml.

Leukocytes. Peripheral blood mononuclear cells were purified from blood collected from healthy volunteers. The monocyte cell line MoNaMc (DSMZ; ACC-124) was obtained from L. Ziegler-Heitbrock, Munich, Germany.

Parasitized red blood cells. Plasmodium falciparum FC83 parasites were cultured on human 0 erythrocytes, in candle jars as described elsewhere (66). They were grown under standard conditions, with replacement of the 10% (vol/vol) human serum with 0.25% (wt/vol) Albumax (Life Technology, Paris, France). Uninfected normal red blood cells (NRBC) used as controls were cultured the same way for at least 2 weeks before experiments. Trophozoite-stage PRBC preparations were enriched to 80 to 85% by gelatin flotation with Plasmon (Fresenius Kabi France, Courrier, France) as described elsewhere (36).
Permeability and resistance alteration measurements in the presence of PRBC. For both techniques, cocultures were carried out as following: HBEC-5i cells were seeded (40,000 to 50,000 cells per well) on polycarbonate filter-etched membrane of 3-μm pores (MilliCell; Millipore, Billerica, Mass.) in 24-well culture plate inserts of 12-mm diameter and grown to confluence. Medium was changed every day in the wells, and then TNF (50 ng/ml; TEBU), LT-α (50 ng/ml; Sigma), or paclitaxel, a strong inducer of apoptosis acting as a mitotic inhibitor (Taxol, 10 nM and 100 nM; Sigma), was added overnight before cocultures were carried out. HBEC-5i cells were then washed in PBS before purified platelets (ratio of platelets to EC, 300:1) or medium was added. Cells were incubated for 90 min at 37°C and then washed in PBS, to remove unbound platelets, and either new medium, NRBC, or PRBC for 90 min (ratio of PRBC to EC, 50:1) at 37°C were then added. EC monolayers were then vigorously washed in PBS to remove bound cells, new medium was added, and HBEC-5i cells were then incubated for 48 h. (i) TEER. EC barrier transendothelial electrical resistance (TEER) was then measured with a Millicell ERS instrument (Millipore). After a 30-min baseline period, TEER was determined every 20 s for the first 2 min and then at 1-min intervals for the subsequent 4 min. Shown here are TEER values at 48 h. The data were corrected for the resistance of the insert alone. (ii) Permeability. For HBEC-5i monolayer permeability assessment, medium was replaced by medium without PBS 24 h before permeability analysis. The study was initiated by adding 1-mM FLITC-labeled dextran (molecular weight, 70,000; Sigma) in DME medium in the insert (in a volume of 100 μl) after three PBS (pH 7.2, 37°C) washing steps. The top chamber was removed and attached to a fresh bottom chamber containing 0.7 ml of fresh DME medium. Several aliquots were harvested from the bottom chamber 15 min after the addition of FLITC-labeled dextran from the top chamber was removed and attached to a fresh bottom chamber containing 0.7 ml of fresh DME medium. Several aliquots were harvested from the bottom chamber 15 min after the addition of the dextran and were transferred to a 96-well plate. HBEC-5i cells were kept at 37°C during the experiment. The diffusion of FITC-labeled dextran from the top to the bottom chamber was then assessed by measuring the fluorescence of aliquots in the 96-well plate with a fluorimeter (FL600; Bio-Tek, Winooski, Vt.), expressed as means ± standard deviations of individual experimental groups. A value of $P < 0.05$ was considered significant.

RESULTS

Morphological characteristics of HBEC-5i cells. The endothelial cell nature of selected pSVT antigen-transfected HBEC-5i cells was confirmed by their capacity to readily adhere to plastic substrate, to form a flat monolayer after being plated in culture flasks (Fig. 1A), to present a thin cobblestone-like morphology after 3 days in culture, and to exhibit contact inhibition. In resting conditions, the HBEC-5i monolayer appeared thin and spread under scanning electron microscopy (Fig. 1B), whereas TNF- or LT-α-stimulated HBEC-5i cells presented a typical activated-EC monolayer pattern composed of elongated and fusiform cells (Fig. 1C and D). To investigate the presence of typical endotheilal features, resting EC were cultured as described above and prepared for transmission electron microscopy analysis. HBEC-5i cells presented a low number of surface villi, with a sparse distribution (Fig. 1E, arrows). The presence of numerous pinocytotic vesicles and Weibel-Palade bodies in the cytoplasm was also revealed (Fig. 1F, arrowheads and “w,” respectively), and HBEC-5i cells exhibited a high number of electron-dense tight junctions (Fig. 1G and H, arrowheads).

Statistical analysis. Statistical analysis was performed with Prism 4.0 from GraphPad, Inc. Data were analyzed by Mann-Whitney U test to compare pairs of groups, except for the TEER and permeability induction results, where data were analyzed by the Kruskal-Wallis and Dunn pairwise tests. Results were expressed as means ± standard deviations of individual experimental groups. A value of $P < 0.05$ was considered significant.
view, see reference 22), the latter two were shown to be markedly up-regulated by TNF. The expression of CD40 was weakly induced by both TNF and LT-α. Other endothelial molecules, such as CD31, CD36, and CD62E, were not detected on the surface of resting or stimulated HBEC-5i cells (Table 1). As shown by slide immunofluorescence, VE-cadherin was poorly expressed in basal conditions (Fig. 2) and not modified by stimulation (not shown). Resting HBEC-5i cells readily expressed von Willebrand factor, a typical endothelial marker (3), and exhibited a peripheral pattern of occludin expression, as shown by immunofluorescence staining (Fig. 2). Occludin is a constituent of endothelial tight junctions, and despite the fact that they are crucial for the endothelial permeability in vivo, these structures were not described in cultured human brain EC so far. Regarding our study, and in terms of endothelial Plasmodium falciparum and platelet receptors, resting HBEC-5i cells were also shown to constitutively express ICAM-1 and CSA and then CD40, respectively. To assess the blebbing and the shedding of MP from the HBEC-5i surface upon cytokine stimulation, cells were stimulated with TNF or LT-α for 6 h and prepared for scanning electron microscopic analysis. Unstimulated HBEC-5i cells presented a smooth surface, with a very low number of vesicles (Fig. 1A and B), while after stimulation (Fig. 3A), cells exhibited a blebbly surface, due to a marked increase in the number of MP. At a higher magnification (Fig. 3B and C), these vesicles showed different diameters ranging from 1 to 2.5 μm (arrows). We therefore quantitated the effects of TNF and LT-α on vesiculation by flow cytometry analysis, and HBEC-5i cells were shown to produce signif-

### TABLE 1. Flow cytometric phenotyping of HBEC-5i cells under resting and activated conditions

<table>
<thead>
<tr>
<th>CD</th>
<th>Antigen</th>
<th>Resting</th>
<th>TNF (10 ng/ml)</th>
<th>LT-α (30 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MFI (AU)</td>
<td>MFI (AU)</td>
<td>MFI (AU)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% Pos</td>
<td>% Pos</td>
<td>% Pos</td>
</tr>
<tr>
<td>IgG1</td>
<td></td>
<td>0.23</td>
<td>0.25</td>
<td>0.28</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>0.24</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>CD36</td>
<td>GPIV</td>
<td>0.26</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>CD51</td>
<td>oxB3</td>
<td>0.23</td>
<td>3.5</td>
<td>0.43</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>9.9</td>
<td>96.3</td>
<td>53.6</td>
</tr>
<tr>
<td>CD62E</td>
<td>E-selectin</td>
<td>0.24</td>
<td>1.3</td>
<td>0.22</td>
</tr>
<tr>
<td>CD106</td>
<td>VCAM-1</td>
<td>5.1</td>
<td>81</td>
<td>11.7</td>
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</tbody>
</table>

*EC were activated with TNF (10 ng/ml) or LT-α (30 ng/ml) and stained by indirect immunolabeling after 24 h, except for CD62E, which was studied after 6 h. Results are representative of a series of three experiments and show both the MFI expressed in arbitrary units (AU) of the labeling with specific monoclonal antibodies and the percentage of cells positively labeled with these antibodies in the whole population.*

![FIG. 2. Immunofluorescence analysis of endothelial markers on HBEC-5i subconfluent monolayers. The figure shows evidence for the expression of several typical endothelial markers on HBEC-5i surface or submembrane, ICAM-1, VE-cadherin, vWF, and occludin. The presence of other molecules with a role in cell-cell interactions, CD40 and CSA, was also demonstrated. Magnification, ×600.](http://iai.asm.org/ on August 29, 2017 by guest)
PRBC cytoadherence was significantly increased over the cytoadherence without platelets ($P < 0.05$, $P < 0.01$, and $P < 0.001$ for resting EC, LT-α, or TNF stimulation, respectively; Fig. 3F) and reached up to $1,758.4 \pm 161.6$ PRBC/mm$^2$ upon TNF stimulation. No statistical difference of the PRBC binding was observed when platelets were added after PRBC on HBEC-5i cells compared to PRBC alone. These results indicate that the phenomenon described for simian cells is reproducible with human-derived brain EC.

Alterations of HBEC-5i confluent monolayers were then investigated after prestimulation by TNF or LT-α and after coculture with platelets, NRBC, PRBC, or a combination of platelets and PRBC. Negative and positive controls were performed with culture medium and NRBC and two paclitaxel concentrations, respectively. We first measured the variation of TEER for each culture condition (Fig. 4A). As expected, both TNF and LT-α caused a significant decrease of the TEER ($P < 0.05$). When HBEC-5i cells were cocultured with PRBC or platelets, this effect was significantly potentiated, with a marked fall of the TEER, from $121.8 \pm 18.6$ Ω/cm$^2$ to $49.8 \pm 9.2$ Ω/cm$^2$ ($P < 0.001$) and $61.7 \pm 6.3$ Ω/cm$^2$ ($P < 0.01$), respectively. However, the most dramatic drop in TEER was observed when TNF- or LT-α-prestimulated HBEC-5i cells were cocultured with platelets followed by PRBC ($P < 0.001$ and $P < 0.01$, respectively; Fig. 4A). In these conditions, the TEER decreased to the level observed with the two doses of paclitaxel, namely, $57.4 \pm 10.8$ Ω/cm$^2$ for LT-α-prestimulated HBEC-5i cells and $16.3 \pm 10.8$ Ω/cm$^2$ for TNF-activated cells. These levels were significantly lower than the value observed with PRBC or platelets alone ($P < 0.01$ for both culture conditions and upon TNF and LT-α stimulation). Moreover, after this sequential coculture, TNF-prestimulated cells exhibited a significantly lower TEER than did LT-activated HBEC-5i cells ($P < 0.05$). Interestingly, when platelets were added after PRBC to activated EC, this dramatic TEER decrease was not observed (Table 2).

Another approach to evaluate the modification of the brain EC barrier function by platelets and PRBC was to investigate the effect of both cell types on endothelial permeability. Using the 70-kDa dextran assay, endothelial integrity was shown to be more affected by a sequential addition of platelets than of PRBC, with a twofold increase in permeability when the monolayer was prestimulated by TNF (Fig. 4B). Once again, the endothelial permeability was significantly more impaired in this sequential coculture condition than with PRBC and platelets alone, and this effect was statistically more pronounced when cells were stimulated with TNF than when they were

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**TABLE 2.** Effect of sequential cocultures of TNF-activated HBEC-5i cells with platelets and PRBC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TNF-activated HBEC-5i cultured with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Platelets</td>
</tr>
<tr>
<td>TEER (Ω/cm$^2$)</td>
<td>128 ± 18.6</td>
</tr>
<tr>
<td>Permeability (optical density)</td>
<td>18.2 ± 4.6</td>
</tr>
<tr>
<td>Apoptosis (% of apoptotic cells)</td>
<td>9.3 ± 2.1</td>
</tr>
</tbody>
</table>

*EC were stimulated with TNF (50 ng/ml) overnight before cocultures. Results are expressed as means ± standard deviations of at least five experiments for each group. Activated HBEC-5i cells alone (−), those cultured with platelets, and those cultured with PRBC are shown as controls. Boldface values indicate a significant difference (Mann-Whitney U test, $P < 0.01$ from values obtained in the condition “PRBC and then platelets.” There were no differences between this latter condition and the condition in which HBEC were cultured with platelets or PRBC alone.*
stimulated with LT-α (P < 0.01). This alteration, similar to that obtained with 100 nM paclitaxel, was not observed when PRBC were added prior to platelets (Table 2). It is worth noting that these effects on HBEC-5i monolayers required the actual binding of platelets, because platelet supernatant alone (collected upon thrombin stimulation) had no effect (data not shown).

Induction of apoptosis by cytokines, platelets, and PRBC.

We investigated the possible induction of HBEC-5i apoptosis in our tripartite coculture model (HBEC-5i cultures, to which platelets and/or PRBC were added) using a quantitation of DNA fragmentation by flow cytometry. Apoptosis was measured 6, 24, and 48 h after cocultures, but significant results were obtained only after a 48-h incubation. It was found that either PRBC or platelets alone potentiated death by apoptosis in TNF-exposed HBEC-5i cells, with an increase of the percentage of apoptotic cells from 9.3% to 18.2% and 22.7%, respectively (Fig. 5A; Table 2). To evaluate the possible effects of leukocytes, increasing numbers of PBMC or MonoMac6 cells were added to HBEC-5i cells in the same conditions of TNF pretreatment. Using HBEC-5i/leukocyte ratios between 1:1 and 1:10, the levels of endothelial killing remained under 25%, 48 h after cocultures, indicating that leukocytes do not significantly alter endothelial viability (S. C. Wassmer et al., unpublished data).

The most drastic effect also was obtained when HBEC-5i cells, following stimulation by TNF, were sequentially cocultured first with platelets and then with PRBC. The percentage of apoptotic cells then reached up to 44.2%. When the sequence of cell addition to the culture was different, i.e., when platelets were added after PRBC, the induction of apoptosis was not significantly different from that observed with platelets or PRBC alone (Table 2). Similar experiments were performed with LT-α pretreatment. No significant increase of the percentage of apoptotic HBEC-5i cells was observed when LT-α-stimulated HBEC-5i cells were cultured with either platelets or PRBC (7.2% to 8.1% and 8.7% to 10.4%, respectively). Upon LT-α stimulation, the addition of both platelets and PRBC led to only 25.5% of HBEC-5i cells becoming apoptotic. When zVAD-fmk was added to the cocultures prior to TNF activation, the joint effect of platelets and PRBC was abrogated, and the HBEC-5i apoptosis rate decreased from 38.1% to 16.2% (Fig. 5B). As controls, NRBC were added to the HBEC-5i cells according to the same timing as the PRBC. These induced no significant increase of apoptosis of resting or TNF- or LT-α-stimulated HBEC-5i cells (4.8, 6.9, and 7.2% of apoptotic cells, respectively; data not shown).

**DISCUSSION**

In this paper, we demonstrated deleterious consequences of both platelet and PRBC adhesion on HBEC-5i monolayer integrity, as well as a potentiating effect of platelets. These effects included increased permeability, decreased TEER, and enhanced apoptosis.

Human-derived cerebral microvascular EC, originally isolated, transected, and stabilized, as the HBEC-5i cell line, by Dorovini-Zis and colleagues (18), exhibited a typical cobblestone-like monolayer at confluence and junctional structures revealed by electron microscopy, a feature relevant to the study of CM pathogenesis. To confirm the endothelial nature of these cells, we showed that HBEC-5i cells presented typical intracellular and surface endothelial features, such as the presence of Weibel-Palade bodies and the expression of von Willebrand factor, VE-cadherin, and occludin (12, 22). Besides, this cell line also was shown to express major proinflammatory cytokine-induced endothelial adhesion molecules such as VCAM-1 and ICAM-1 (62), but not CD31 and CD36. While CD31 is a molecule involved in cell-cell junctions, the HBEC-5i line described here still presented major features of cerebral EC, especially efficient tight-junction structures as assessed by high TEER and low permeability. Moreover, several studies with mice showed that CD31 deficiency in mice has no repercussions on animal viability: they remain healthy and do not exhibit obvious vascular developmental defects, leukocyte migration impairment (19), or vascular alteration (73). Also, CD31 is frequently lost in EC lines whereas it is expressed on primary cells (71, 72). The absence of CD36 on HBEC-5i cells is consistent with previous data (2, 61, 70) showing a low and irregular CD36 expression among brain microvessel walls. In a more recent study, CD36 was not found in brain microvessels, except in children who died from CM; in this case, the CD36 labeling appeared to be restricted to bound platelets (29). This distinctive characteristic can be relevant to modeling specific brain microvascular lesions, especially those...
observed during CM. Indeed, we recently have demonstrated the relevance of an in vitro endothelial model using CD36-deficient SBEC Sc1707 (23) for CM lesion analysis (75). However, these cells had been derived from Saimiri sciureus monkeys, and an in vitro model based on human brain microvessels may be more suitable to study pathogenic mechanisms of human CM. Furthermore, since HBEC-5i cells express various parasite cytoadherence receptors, such as ICAM-1, VCAM-1, and CSA, this cell line could be a relevant tool to better understand pathological processes such as cerebral, pulmonary, or placental lesions of severe malaria.

Another feature of several EC lines is the increased release of MP in response to vesiculation agonists such as TNF (14, 40, 41). Here we showed both by electron microscopy and by flow cytometry that HBEC-5i cells are able to release high numbers of MP upon TNF and LT-α stimulation compared to resting conditions, as well as in the presence of platelets. The shedding of MP could be of pathological interest since we recently demonstrated the presence of high numbers of endothelial MP in the plasma of Malawian children, specifically during the acute phase of CM compared to the other fatal complication of malaria, severe anemia (15). This could be related to the massive accumulation of platelets within brain microvessels of Malawian children who died of CM, as we provided evidence for a provesculation effect of platelets on EC. Moreover, since it has been shown in vitro that MP carry at their surface the same adhesion antigens as their cell of origin, one can hypothesize that in vivo they will disseminate in the circulation, creating new adherence sites for PRBC. Thus, HBEC-5i-derived MP will represent a valuable new tool to better understand the role of MP in the pathogenesis of the cerebral syndrome. We also showed a platelet-induced increase of PRBC cytoadherence, a finding consistent with the phenomenon described recently in vitro with simian brain EC (75), reinforcing the hypothesis of a crucial role for platelets in human CM pathogenesis.

Resting confluent HBEC-5i monolayers exhibited a high electrical resistance and a low permeability to 70-kDa dextran, two characteristics of the presence of functional tight junctions. Indeed, permeability of the EC barrier largely depends on the restriction to fluid transport across the paracellular pathway that contains tight and adherens junctions (34), which are the primary determinants of barrier function (25, 38, 39, 43, 57, 68). When platelets were added prior to PRBC on TNF- or LT-α-activated HBEC-5i cells, both the electrical resistance and 70-kDa dextran permeability of HBEC-5i monolayers were dramatically affected compared to the effect of the cytokines alone. Indeed, the endothelial barrier displayed a complete loss of electrical resistance and, conversely, the permeability drastically increased. Those two related cytokines are involved in CM pathogenesis: both of them are found in high titers in the plasma of CM patients, and they interact with a common receptor, TNFFR2, which has been shown to play a key role in the microvascular lesion (37). Moreover, it was recently shown that LT-α-knockout mice are protected against CM induced by Plasmodium berghei ANKA, whereas TNF-knockout mice are not, suggesting that LT-α could be the important molecule in the pathogenesis of murine CM (21). Although there is evidence consistent with TNF having a crucial role in the pathogenesis of CM (13, 30, 32), both TNF and LT-α may have various degrees of involvement in the microvascular lesion, as suggested here in our human CM in vitro model based on brain endothelium.

The occurrence of this endothelial apoptosis as a consequence of platelet and PRBC adhesion might result in BBB dysfunction and could amplify the pathological process. Indeed, the presence of apoptotic cells might up-regulate the expression of cellular adhesion molecules on living EC in the vessel wall, resulting in their hyperadhesiveness (35).

The sequence of events thus appears to be critical for EC alterations, since platelets had no effects if they were added after PRBC. In view of our coculture results, we may hypothesize that EC apoptosis induced by PRBC is aggravated provided that these EC have undergone a prior exposure to platelets. This potentiation is likely to be involved in the loss of endothelial integrity. Indeed, we recently provided evidence for a strong proapoptotic effect of PRBC-activated platelets on inflamed endothelium. Platelet activation leads in this context to the release of transforming growth factor β, which acts as an inducer of apoptosis of activated HBEC-5i cells (75a). This, added to the previously described proapoptotic effect of PRBC (53), could explain in vivo the significant BBB functional alteration and the subsequent transit of plasma proteins and fluid into the perivascular space and brain parenchyma, contributing to cerebral edema observed during CM (52, 56, 59).

Proposals that the BBB might be damaged during CM date back to Maegraith’s work in the 1940s (46). Recent evidence has strengthened this link (10). Histological, immunohistochemical, and ultrastructural studies of postmortem brain tissues, first performed with the mouse model (50, 65), then more recently in fatal cases of human CM, have revealed structural changes at the BBB (9, 10). These include immunohistochemical evidence of widespread induction of endothelial activation antigens, with significantly higher levels of ICAM-1 and E-selectin expression on vessels in the brain (56, 61, 70).

Disruption of interendothelial junctional proteins in CM also has been observed ex vivo. Immunohistochemistry of postmortem tissue from Vietnamese adults (9) and Malawian children (10) revealed a loss of EC junctional proteins ZO-1, occludin, and vinculin, most notably in vessels containing sequestered PRBC. Moreover, hemorrhages resulting from a BBB breakdown are commonly observable in brain tissue from fatal malaria cases (69) and in the mouse model (27, 50, 65). These results provide clear evidence of focal BBB leakage, especially in vessels showing PRBC sequestration through adhesion to EC. Also, even if platelets in vitro did not have a deleterious effect on EC when added after PRBC, they could represent in vivo the final step of a pathological process initialized by PRBC sequestration and leading to microvessel occlusion.

Taken together, the data presented here are the first demonstration of the pathological contribution of platelets in an in vitro model of human CM, acting together with PRBC in causing EC alterations. In a mouse model of CM, it had been shown that platelet deposition appears to be a major contributor to death, given that platelets accumulate in microvessels and that antiplatelet therapy (28, 31) as well as platelet adhesion inhibition (64) can improve outcome. Moreover, in Malawian children who died of CM, platelet accumulation was strongly associated with fatal outcome (29). While platelets were recently shown to be involved in PRBC sequestration by provid-
ing new parasite receptors or by hiding expressed receptors on EC (75), their cooperating effect with PRBC on the endothelial integrity impairment during CM has not been reported before.

In conclusion, the present study performed with the HBECS-5i line demonstrates that the sequential presence of platelets and PRBC has a potentiating effect on stimulated HBECS-5i cells. Enhancement of permeability and potentiation of apoptosis appear to be important mechanisms by which platelets can alter EC. These data provide evidence that, in addition to their adhesion, demonstrated in vivo and in vitro, platelets can have a pathogenic role in this CM model based on brain endothelium. Moreover, our data suggest that these aspects of EC alterations are specifically induced by TNF or by LT-α in vitro. A better understanding of the complex interactions between platelets, PRBC, and the endothelium leading to vascular injury during CM is necessary to develop new therapeutic approaches to improve the outcome of the disease.

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