Platelet-Induced Clumping of *Plasmodium falciparum*—Infected Erythrocytes from Malawian Patients with Cerebral Malaria—Possible Modulation In Vivo by Thrombocytopenia

Samuel Crocodile Wassmer,1,2* Terrie Taylor,3,5 Calman Alexander MacLennan,1,6 Maxwell Kanjala,1 Mavuto Mukaka,1 Malcolm Edward Molyneux,1 and Georges Emile Grau4

1Malawi-Liverpool-Wellcome Trust Clinical Research Programme and 2Department of Haematology, College of Medicine, University of Malawi, Chichiri, and 3Blantyre Malaria Project, Blantyre, Malawi; 4Vascular Immunology Unit, Department of Pathology K25 and Bosch Institute, Faculty of Medicine, The University of Sydney, Sydney, Australia; 5Department of Internal Medicine, College of Osteopathic Medicine, Michigan State University, East Lansing, Michigan; 6Centre for Immune Regulation, Division of Immunity and Infection, The Medical School, University of Birmingham, Birmingham, United Kingdom

Platelets may play a role in the pathogenesis of human cerebral malaria (CM), and they have been shown to induce clumping of *Plasmodium falciparum*—parasitized red blood cells (PRBCs) in vitro. Both thrombocytopenia and platelet-induced PRBC clumping are associated with severe malaria and, especially, with CM. In the present study, we investigated the occurrence of the clumping phenomenon in patients with CM by isolating and coincubating their plasma and PRBCs ex vivo. Malawian children with CM all had low platelet counts, with the degree of thrombocytopenia directly proportional to the density of parasitemia. Plasma samples obtained from these patients subsequently induced weak PRBC clumping. When the assays were repeated, with the plasma platelet concentrations adjusted to within the physiological range considered to be normal, massive clumping occurred. The results of this study suggest that thrombocytopenia may, through reduction of platelet-mediated clumping of PRBCs, provide a protective mechanism for the host during CM.

Severe malaria is a major cause of death in sub-Saharan African children. A pathological characteristic that is common to the diverse life-threatening syndromes that can result from *Plasmodium falciparum* infection is the sequestration of large numbers of mature parasitized red blood cells (PRBCs) within the microvasculature of vital organs. Sequestration is generally attributed to cytoadherence of PRBCs on microvascular endothelial cells [1]. However, postmortem studies have shown that intravascular PRBCs are not adjacent to the vascular endothelium [2], suggesting that some PRBCs may be adherent to each other rather than to the vascular endothelium and that they might have accumulated through a process of adhesion between PRBCs.

Platelet accumulation can be found in the cerebral microvessels of patients who died of cerebral malaria (CM) but not in those of patients who died of severe malarial anemia (SMA), suggesting a possible role for platelets in the pathogenesis of cerebral CM [3]. Platelets can mediate the formation of PRBC clumps in vitro [4]. This phenomenon has been shown to be more common when parasites from patients with severe malaria, rather than isolates from patients with mild malaria, are used, both in Kenyan children [4] and in Thai adults [5]. The size of the clumps observed in vitro is sufficient to be responsible for important rheological disturbances in infected patients, if the clumps are similarly sized in vivo.

Thrombocytopenia is a typical characteristic of *P. falciparum* infection. The degree of thrombocytopenia was found to correlate with the risk of a fatal outcome in one study [6] but not in another [7]. We postulated that the
presence of abnormally low numbers of circulating platelets in patients with malaria would restrict the formation of platelet-RBC aggregates, an effect that might limit the severity of cerebral pathology.

**PATIENTS AND METHODS**

**Patients.** We studied consecutively observed cases of uncomplicated malaria (UM), SMA, and CM in patients who were admitted to the pediatric research ward of the Queen Elizabeth Central Hospital in Blantyre, Malawi. The clinical characteristics of these patients are detailed in table 1. All patients with UM and SMA had *P. falciparum* parasitemia and were conscious (Blantyre coma score, 5 of a possible 5). These patients were considered to have UM if the packed cell volume was >25\% and SMA if it was <12\%. Patients with CM were admitted to the hospital in a coma (Blantyre coma score, =2), had *P. falciparum* parasitemia, and had no other clinically evident cause of unconsciousness. All of the patients with CM who were included in the present study showed evidence of malarial retinopathy, a recently described finding that improves the accuracy of the clinical diagnosis of CM (for review, see [8]). The patients’ relatives provided fully informed consent to participate in the study, and a 2.5-mL blood sample was taken for parasite culture and separation of plasma and platelets. The studies were approved by the ethics review committees of the College of Medicine, University of Malawi (Blantyre); the Liverpool School of Tropical Medicine (Liverpool, United Kingdom); and Michigan State University (East Lansing).

**Parasite culture.** Blood samples from patients were collected in sodium citrate tubes and centrifuged at 250 g for 10 min. Plasma was stored at 4°C, and pelleted RBCs were washed 3 times in RPMI 1640 and then were resuspended in a standard malaria culture medium of RPMI 1640 supplemented with 25 mmol/L HEPES, 10\% fetal calf serum, and 40 mg/mL gentamicin, to achieve a final hematocrit of 5\%. After cultivation at 37°C in 5\% CO₂ for up to 48 h, the parasite stages and numbers were adjusted to a 10\% mature-form (the pigmented trophozoite stage) parasitemia by means of gelatin flotation, to standardize the assays and compare the results. Blood smears were then prepared, and the stage of parasite maturation was examined by microscopy.

**Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP).** PRP and PPP were extracted from 5 mL of whole blood provided by malaria-naive donors or from 2.5 mL of whole blood obtained from patients with CM. Blood was collected in a sodium citrate tube and centrifuged at 250 g for 10 min. The PRP fraction was then transferred to a new tube, and a platelet count was performed with Neubauer’s hematocytometer before storage. PPP was obtained by further centrifugation performed at 1500 g for 10 min to discard platelets, and both PRP and PPP were stored at 4°C and used within 4 days.

**Clumping assays.** Clumping kinetics (figure 1A) were assessed when the parasites had grown to the stage of pigmented trophozoites and were selected as described above. Parasite cultures labeled by the addition of 20 mg/mL acridine orange were then rotated in 5\% hematocrit in the presence of 20\% PPP (platelet count, <10 platelets/μL) or 20\% PRP (platelet count, >300 × 10³ platelets/μL [for healthy donors] or <150 × 10³ platelets/μL [for patients with CM]). After 15 min of rotation, 25 μL of sample was obtained, placed on a glass slide, and examined by fluorescent microscopy. Additional samples were obtained at 30, 60, and 120 min, as described elsewhere [4]. A “clump” was defined as ≥3 infected erythrocytes, and the frequency of the clumping phenotype in isolates was measured as the number of infected cells in clumps among 1000 infected cells counted in duplicate assays. For the autoassays (figure 1B), PRP, PPP, and cultured parasites isolated from the same patient with CM were used (in vivo platelet count, <150 × 10³ platelets/μL). Platelet numbers were concentrated 5 times in plasma samples used for autoassays, so that, when mixed with parasite suspensions, the final concentration of platelets would be representative of the in vivo conditions. Platelet concentration was accomplished by gentle centrifugation, removal of a calculated portion of plasma, and resuspension of platelets. To assess the potential influence of plasma factors on clump formation, the same technique was used to adjust several platelet suspensions to a final average

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with UM (n = 12)</th>
<th>Patients with SMA (n = 15)</th>
<th>Patients with CM (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (IQR), months</td>
<td>55.5 (29–82.5)</td>
<td>45 (30–82)</td>
<td>36.5 (18–63)</td>
</tr>
<tr>
<td>Blantyre coma score, median (range)</td>
<td>5</td>
<td>5</td>
<td>1 (0–3)</td>
</tr>
<tr>
<td>Parasitemia, geometric mean (95% CI), parasites/μL</td>
<td>23,617 (8910–62,560)</td>
<td>99,550 (63,400–156,300)</td>
<td>68,700 (27,750–171,300)</td>
</tr>
<tr>
<td>Platelet count, median (IQR), ×10³ cells/μL</td>
<td>67 (37.5–232.5)</td>
<td>97.2 (72–124)</td>
<td>58 (21–145.5)</td>
</tr>
<tr>
<td>Spearman’s correlation testb (P value)</td>
<td>0.3916 (0.5)</td>
<td>0.1055 (0.5)</td>
<td>−0.675 (&lt;.001)</td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; CM, cerebral malaria; IQR, interquartile range; SMA, severe malaria anemia; UM, uncomplicated malaria.

a Thrombocytopenic groups: a patient was considered to have thrombocytopenia when his/her platelet count was <150 × 10³ cells/μL.

b For assessment of the correlation between the platelet count and parasitemia.
Figure 1. Schematic representation of clumping assays. Classic clumping assays were performed as described elsewhere [4, 5], with the use of parasites that were isolated from infected patients, cultured, and incubated with platelet-poor plasma (PPP) and platelet-rich plasma (PRP) obtained from healthy donors with a normal platelet count (A). In autoassays, parasites, PPP, and PRP were all obtained from the same patient, to assess the potential for occurrence of this phenomenon ex vivo (B). CM, cerebral malaria; SMA, severe malarial anemia; UM, uncomplicated malaria.

Analysis of platelet activation in clumps. Variation of platelet membrane P-selectin expression was measured by immunofluorescence on slides and by flow cytometry. Both were performed on platelets alone in PRP obtained from patients with CM, as well as on clumps after 2 min of coincubation of PRP with PRBCs. For the immunofluorescence studies, PRP alone or PRP after coincubation in clumping assays was incubated at room temperature for 20 min with fluorescein isothiocyanate–coupled mouse anti–human CD62-P (P-selectin, clone JMCD62P; Orbigen) before observation with the use of a fluorescence microscope. Flow cytometric analyses required clumps of small or medium size, and the antibody was added, either 5 min before PRP analysis or after 2 min of PRP-PRBC coincubation, for 5 min. In the latter case, the total of 7 min of coincubation led to the formation of clumps of the desired dimension.

Inhibition of platelet-induced clumping by monoclonal antibodies. PRP samples were incubated at 37°C for 1 h with several blocking antibodies against platelet membrane glycoproteins, such as CD36 (GPIV, clone FA6-152; Beckman Coulter Immunotech), P-selectin (CD62-P, clone AK-6; Serotec), GPIIb/IIIa (CD41/CD61 chimeric antibody, clone 7E3 [ReoPro; Centocor]; a gift from G. Wiemann), or IgG isotype control. Lamifiban (Ro44-9883; Roche) (provided by Jürgen Fingerle of F. Hoffmann–La Roche), a peptidomimetic synthetic GPIIb/IIIa inhibitor, was also incubated with PRP for the same length of time. PRP was then used in clumping assays performed as previously described.

Statistical analysis. Statistical analyses were performed using Stata software (version 8.1; Stata). Data were analyzed using the Mann-Whitney U test to compare pairs of groups. Results were expressed as the mean value (±SD) for individual experimental groups, and the clumping data for the different patient groups were analyzed using the Kruskal-Wallis test and Dunn’s pairwise test. The correlation between parasite density and platelet count in table 1 was assessed using Spearman’s correlation test. P < .05 was considered to be statistically significant.

RESULTS

Association of platelet-induced PRBC clumping phenotype in children with severe malaria in Malawi. We assessed the occurrence of the previously described platelet-induced in vitro PRBC clumping phenomenon in Malawian children with *P. falciparum* malaria. Parasites isolated from 49 patients were studied; 12 of these 49 patients had UM, and, of the 37 patients who had severe malaria, 15 had SMA and 22 had CM (table 1). In the presence of PPP from healthy donors, no PRBC autoagglutination was observed in any of the patient groups after coculture for 120 min (figure 2A–2C). When incubation with PRP was performed, however, the platelet-induced clumping phenotype was observed in all groups. This phenomenon was time dependent and was associated with disease severity and type. Parasites isolated from patients with UM showed a weak ability to agglutinate in the presence of platelets, with 27.1% ± 12.0% of clumped PRBCs present at the end of the kinetics studies.

Microscopic analysis showed, for cases of UM, a homogeneous suspension of PRBC, with some small clumps (8.6 ± 3.0 PRBCs/clump) randomly distributed (figure 2A). For cases of SMA or CM, autoagglutination was significantly higher than that noted for cases of UM (at 60 min and after for SMA [P < .05 at 60 min; P < .001 for all of the other times] and at 15 min and after for CM [P < .001 for each kinetics point]).

For SMA, clumped PRBCs constituted 59.1% ± 14.1% of the total PRBC population at the end of the kinetic study, and significantly larger clumps were observed for SMA than for UM (42.9 ± 9.5 vs. 6.5 ± 4.3 PRBCs/clump, respectively) (P < .001) (figure 2B). In the presence of platelets, parasites isolated from patients with CM exhibited a statistically stronger clumping phenotype than did parasites from patients with SMA (P < .001, for each kinetics point), leading to the formation of giant clumps (>50 PRBCs/clump) within the first 15 min of incubation and to the aggregation of all of the PRBCs in the suspension. The number of PRBCs could not be counted pre-
cisely in giant clumps, but some clumps contained up to 125 PRBCs (figure 2C). In each experiment, we used as controls uninfected erythrocytes isolated from healthy and malaria-naive donors, all of whom exhibited platelet counts within the range considered to be normal (150 × 10^3 to 450 × 10^3 platelets/μL). Cells were incubated with PPP or PRP that was obtained and processed as described in the experimental procedures, and aggregation was never observed (data not shown).

**Involvement of platelet activation and subsequent up-regulation of P-selectin in the clumping effect.** To assess platelet activation during clumping, we used immunofluorescent staining to identify P-selectin on platelets both before and after 10 min of coincubation with PRBCs. Samples were analyzed using fluorescent microscopy and flow cytometry. Before coincubation with PRBCs, platelets obtained from patients with CM did not show any sign of activation: they were of normal circular shape (figure 3A). Flow cytometric analysis showed that the gated platelet population had low P-selectin surface expression, with a mean fluorescence intensity (MFI) of 5.8% (figure 3C and 3E). Within 10 min of the addition of PRBCs, platelets exhibited typical activation pseudopodia (figure 3D), and significant up-regulation of membrane P-selectin was observed for the platelet and clump populations, reaching an MFI of up to 77.4% (figure 3D and 3F). Platelets not associated with clumps (figure 3D, gate [p]) exhibited an increased MFI of 49.9% (data not shown). A control experiment was performed by mixing unparasitized erythrocytes with PRP, and neither platelet changes nor uninfected erythrocyte clumping was observed (data not shown).

To investigate the potential role of platelet P-selectin as a receptor for PRBC in this aggregation phenomenon, PRP was then incubated in the presence of blocking anti–P-selectin, as well as several other antibodies or antagonists against the platelet membrane glycoproteins listed above, before undergoing incubation with PRBCs isolated from patients with CM. In the presence of anti–P-selectin, platelet-induced PRBC clumping was significantly but partially decreased, with 20.7% ± 11.0% inhibition (P < .001), compared with clumping noted for controls (figure 3E). Incubation with anti-CD36 led to a dramatic reduction in aggregation (67.3% ± 6.5%; P < .01), as described elsewhere [4], and a combination of both antibodies led to almost complete abrogation of clumping, with greater inhibition than the additive effect of the antibodies taken separately (97.7% ± 10.5%; P < .01). By contrast, the blocking of the platelet surface glycoproteins GPIIb and IIIa by high concentrations of ReoPro or Lamifiban (1–30 μg/mL and 50–100 ng/mL, respectively), agents that are both known to block platelet aggregation [9], failed to induce significant inhibition of PRBC aggregation (P > .05) (figure 3E). When platelets were inactivated by pretreatment with heparin to inhibit the up-regulation of P-selectin on their surface (1000 U/mL), the same level of inhibition reported for anti–P-selectin treatment was observed (data not shown).

**Figure 2.** Ability to form clumps (defined as >3 parasitized red blood cells [PRBCs]) in the presence of platelets in parasite populations isolated from pediatric patients in Malawi. Platelet-induced PRBC clumping is a common parasite phenotype among Malawian children and is associated with disease severity. Parasites isolated from patients with uncomplicated malaria (n = 12), severe malaria anemia (n = 15), and cerebral malaria (n = 22) were incubated for different times with plasma with or without platelets. Results are expressed as the mean percentage (± SD) of PRBCs associated with clumps. Assays were performed twice for each patient sample.
Restriction of ex vivo clump formation by thrombocytopenia in patients with CM. The occurrence of clump formation ex vivo in the presence of thrombocytopenia was then evaluated by the use of PRP and PRBCs from the same patient with CM. Healthy donors who provided the PRP used in previous assays exhibited an average platelet count of >300 × 10^3 platelets/μL, whereas all 22 patients with CM had thrombocytopenia, with platelet counts of <150 × 10^3 platelets/μL at admission (table 1). When incubated with PRP from the same patient with thrombocytopenia, PRBCs showed a significantly reduced clumping phenotype (mean percentage of reduction [± SD] after a 120-min incubation step, 39.6% ± 19.4%). To assess whether this clumping restriction was due only to the low platelet count and not to other circulating plasma factor(s) in these patients with CM, we adjusted the PRP to a final average platelet count of >300 × 10^3 platelets/μL, by use of a low-centrifugation step as described above. When PRP with an adjusted platelet count was incubated with PRBCs, there was a partial but significant restoration of the clumping, reaching 82.6% ± 11.6% after 120 min (P < .001) (figure 4). A control experiment performed with PRP obtained from healthy donors with a platelet count adjusted to disease-type concentration by dilution (60 × 10^3 platelets/μL, as calculated in table 1) led to a significant decrease in clumping (data not shown).

Correlation of the degree of thrombocytopenia with the density of parasitemia in patients with CM in Malawi. The association between the platelet count and the density of parasitemia was investigated in the 3 patient categories (table 1). A negative coefficient of correlation (−0.675) was found between these 2 variables in the CM group, with Spearman’s correlation test showing strong statistical significance (P < .001). This association was not observed in patients with SMA or UM.

DISCUSSION

In the present study, we demonstrated that platelet-mediated PRBC clumping is a stable and common in vitro phenotype of all of the *P. falciparum* strains isolated from 49 infected Malawian children. We also showed that this phenotype is quantitatively associated with disease severity and type, that the platelet molecules responsible for this effect are CD36 and P-selectin, and that the degree of thrombocytopenia present in patients with CM is sufficient to limit the further formation of PRBC clumps in vitro.

The aims of the present study were, first, to document the occurrence of the platelet-induced PRBC clumping phenomenon in parasites isolated from Malawian pediatric patients and, second, to assess its association with disease severity and type (UM vs. severe malaria, as well as SMA vs. CM). We demonstrated clumping of PRBCs in the presence of PRP for all isolates recovered from patients with malaria who were admitted to the ward, irrespective of their clinical syndrome. The ability to generate clumps in the presence of a standard preparation of plate-
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Adhesion of PRBCs to activated endothelium [11]. Uneven deposition of platelets may contribute to the observed disparate distribution of sequestration in the microvasculature of patients with CM [12].

We then further analyzed the role of platelet activation in the formation of clumps. Previous studies have shown that PRBCs are able to activate platelets [13, 14], leading to the immediate transfer of the internal P-selectin (stocked in the platelet α granules) to its surface [15]. Because the presence of P-selectin has been described as a synergistic factor in the binding of PRBCs on endothelial CD36 [16], we hypothesized that platelet P-selectin was likely to be involved in clump formation. Its expression on platelet membranes increased dramatically during clumping, confirming that platelets are activated by PRBCs. We also demonstrated, by use of inhibition assays, that P-selectin was involved as a PRBC receptor in clump formation.

The other molecule shown to be involved in PRBC-platelet adherence in the present study was CD36. Both P-selectin and CD36 are expressed on platelets, and both are known to be receptors for PfEMP-1, the parasite protein expressed on the surface of PRBCs [17–20]. These findings are consistent with previous studies [4] of CD36, but P-selectin has not previously been identified as a receptor mediating platelet-PRBC clumping. When both CD36 and P-selectin were blocked by antibodies, the clumping phenomenon was abrogated, and our results suggest synergism between the 2 receptors during clump formation.

P-selectin has been reported to increase the adherence of PRBCs to CD36 on endothelium [16], and its substantial but transient expression on PRBC-activated platelet surfaces may act as a trigger for the amplification of the clumping phenomenon. Such a “clumping-activation-clumping” loop might explain the massive aggregation observed in the early minutes of our assays with CM-derived PRBCs. Platelet activation as a consequence of clumping formation in vivo would lead to a massive release of transforming growth factor–β1, which is contained in the platelet α granules. This pleiotropic cytokine has recently been described to act as an inducer of apoptosis in endothelium in inflammatory conditions; platelet activation in sequestration sites in vivo may therefore make additional indirect contributions to pathogenesis [14].

Finally, we investigated the possibility of a natural restriction of clumping associated with thrombocytopenia in patients with malaria. The formation of giant clumps in vivo could be expected to lead to important rheological disturbances and microvessel occlusion, yet in vivo studies have suggested that sequestration of PRBCs is not sufficient to block brain microcirculation in patients with CM [1, 21]. Because platelets seem to be a crucial element in clump generation, we investigated the effect of thrombocytopenia, exhibited by all of our patients with CM (table 1), on clump formation ex vivo. We developed a new method of ex vivo coculture of different cell types (i.e., platelets and PRBCs) isolated from the same patient,
and we compared the effect of PRP from healthy individuals (average platelet count, \(>300 \times 10^3/\mu L\)) with the effect of PRP from the same patient (average platelet count, \(<150 \times 10^3/\mu L\)) on clump formation. Plasma from patients with thrombocytopenia failed to generate giant clumps, and an adjustment of the platelet count to within the range considered to be normal in these plasma samples led to the restoration of the phenomenon. These results suggest that, in patients with malaria, thrombocytopenia may have the potentially beneficial effect of restricting clump formation in vivo, leading to less platelet activation and, thereby, modulating pathogenesis.

The degree of thrombocytopenia in pediatric patients with CM was significantly correlated with parasitemia (Spearman’s correlation test, \(-0.675; P < .001\)) (table 1). A possible explanation for this correlation would be that thrombocytopenia results from widespread clumping in sequestration sites, the degree of which is directly linked to the parasite burden of the patient. However, this hypothesis is not supported by experiments in mice in which abrogation of the platelet trapping in cerebral microvasculature had no effect on the low platelet count of the animals [22]. Whatever the mechanism of thrombocytopenia, it appears that, in human CM, the invariably low platelet count may function as a protective mechanism for the host.

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