Title

Bridging and clumping: investigating platelet interactions with parasitized erythrocytes and endothelial cells in cerebral malaria

Summary

The methods presented in this chapter describe how to perform ex vivo clumping and in vitro bridging assays in the context of cerebral malaria. Both protocols are detailed, and emphasis is made on how to prepare platelet suspensions suitable to each technique, including description of specific buffers and reagents to minimize the risk of aggregation while maintaining the platelet properties.

Key Words

Cerebral malaria, *Plasmodium falciparum*, platelet-rich plasma, platelet-poor plasma, adhesion, clumping, bridging

Introduction

The role of platelets has recently come to light in the development of both human and experimental cerebral malaria (CM and ECM, respectively). First considered innocent bystanders, platelets have emerged as potent enhancers of *Plasmodium falciparum*-parasitized red blood cell (PRBC) sequestration and major players in local blood–brain barrier (BBB) disruption [1].

Their accumulation within cerebral microvessels in fatal CM was first described using electron microscopy [2]. Further analyses of human post-mortem brain sections from fatal pediatric CM, severe malarial anemia, and nonmalarial cases revealed a strong correlation between intravascular platelet accretion and disease severity [3]. These results were in line with previous studies in a murine model of the disease, where platelets were also reported to accumulate in brain microvessels. Not only was this phenomenon abrogated in mice by treatment with an anti-leukocyte function antigen-1 monoclonal antibody [4], a molecule responsible for the binding of platelets to endothelial cells, but the experimental induction of thrombocytopenia prevented the development of ECM and its associated mortality [5].

In Malawian patients who died of CM, accumulated platelets were regularly found to colocalize with malarial pigment on the endothelial surface, suggesting that they interact closely with both endothelial cells and PRBC during the development of the disease [3]. Because platelets constitutively express CD36, one of the major receptors for PBRC [6], it was then postulated that they could modulate the cytoadherence of PRBC onto brain endothelial cells, thereby promoting vascular occlusion [7]. Not only was this hypothesis proven right, but also two distinct platelet-dependent mechanisms of sequestration enhancement, namely bridging and clumping, were described.

Platelets can bind to tumor necrosis factor (TNF)-activated endothelial cells, and through their surface CD36, are then able to form bridges between PRBC and cerebral endothelium [8]. This

bridging was observed *in vitro*, both in static and flow conditions. Remarkably, it can also occur when platelets bind strings of von Willebrand Factor (vWF) multimers attached the endothelial surface [9]. In addition, platelets can mediate the clumping of PRBC ex vivo, a phenomenon that that was associated with severe malaria in patients from Kenya [10], Thailand [11], Malawi [12] and Mozambique [13]. Conversely, a study in Mali found an association with high parasitaemia, but not severe disease [14]. The molecular mechanisms underlying the interactions of PRBC with platelets are not fully understood; however, three platelet receptors for clumping have been identified to date: CD36 [10]; the globular C1q receptor (gC1qR/HABP1/p32) [13]; and P-selectin [12]. While the formation of clumps has been reported in vitro and ex vivo, it is probable that this phenomenon would contribute significantly to the vascular obstruction observed in CM, should it occur in vivo. The platelets consumption resulting from both bridging and clumping may, in turn, contribute to the commonly observed thrombocytopenia in CM, a feature recently associated with brain swelling identified by magnetic resonance imaging in pediatric patients from Malawi [15]

Understanding the molecular interactions between platelets, PRBC and brain endothelial cells in the context of CM will provide new insights in the pathology of the neurological syndrome and, ultimately, might open new therapeutic approaches. However, discrepancies in clumping findings have been attributed to different experimental conditions, which need to be standardised for comparable results across malaria-endemic sites [16]. We have therefore developed protocols to investigate and decipher the role of platelets in both clumping and bridging, using specific ex vivo and in vitro approaches described in this chapter.

1 - Materials

1.1 Clumping

Equipment:

- Sodium citrate (3.2-3.8%) blood collection tubes, preferably evacuated
- Tourniquet and phlebotomy set
- Centrifuge
- Waterbath or incubator set to 37°C
- Pipettes: 20 and 200µl
- Electronic pipette controller, 5 and 10ml plastic pipettes
- Haematocytometer (Malassez or Neubauer) with coverslips
- Automatic tube roller
- 0.5 mL cryotubes
- Parafilm
- Microscope slides
- Coverslips
- Fluorescent microscope
- For parasite culture, see Chapter XX on general parasite culture

Reagents:

- Acridine orange
- PBS
- For parasite culture, see Chapter XX on general parasite culture

1.2 Bridging

Equipment:

- Sodium citrate (3.2-3.8%) blood collection tubes, preferably evacuated
- Tourniquet and phlebotomy set
- Temperature-controlled centrifuge set to 22°C and pre-warmed if necessary.
- Waterbath set to 37°C
- Incubator set to 37°C
- Pipettes: 20 and 200μl
- Electronic pipette controller, 5 and 10ml plastic pipettes
- Hematocytometer (Malassez or Neubauer) with coverslips
- 12-well immunofluorescence assay (IFA) slides
- Plastic Petri dishes
- Coverslips
- Inverted microscope
- For parasite culture, see Chapter XX on general parasite culture
- For endothelial cell culture, see Chapter XX on general endothelial culture for binding assays

Reagents:

- Suspended PRBC (trophozoite or schizonts) from culture (see Chapter XX on general parasite culture)
- Confluent cultured human brain endothelial cells (see Chapter XX on general endothelial culture for binding assays)
- Recombinant human TNF (Sigma)
- Tyrode's buffer, as described below, pH adjusted to 6.9 (see Table 1)

- Prostaglandin I2 (PGI_2, Sigma)
- Heparin sodium 25000 I.U./ml solution for injection
- Paraformaldehyde-lysin-periodate (PLP, see Table 2)

2 - Methods

2. 1 Clumping

1. *Blood samples.* Collect blood in vacutainers containing 3.8 M buffered sodium citrate as anticoagulant (ratio 1: 9).

2. *Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) preparation.* Centrifuge blood samples immediately after collection at 250 *x g* for 10 min **(Fig. 1A)**. Carefully collect the cloudy yellow top PRP layer and store at 4C until PRBC are ready to use **(Fig. 1B)**. For controls, a fraction of the PRP can be further centrifuged for 10min at 2,000 *x g* to pellet platelets and generate platelet-poor plasma. Assess platelet counts in both PRP and PPP using a hematocytometer (*see* **Note 1**).

3. *Short blood culture*. See Chapter XX on general parasite culture for additional details. Briefly, wash pelleted RBC remining from Step 2 above 3 times in pre-warmed RPMI-1640, then resuspend 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 up to 48 h of cultivation at 37°C in 5% CO_2 **(Fig. 1C)**, adjust the parasite stages and numbers to a 10% mature form (pigmented trophozoites) parasitemia by gelatine flotation or MACS purification (see Chapter XX on general parasite culture).

4. *Clumping assays*. To evaluate clumping phenotypes and kinetics, label parasite cultures by addition of 20 mg/ml acridine orange. Add the labelled parasite suspension (5% hematocrit) to a clean cryotube tube and mix gently with 20% PPP or 20% PRP to avoid platelet activation. Seal each cryotybe with Parafilm (*see* **Note 2**) and place on an automatic roller on the lowest rotation setting (10-20rpm, *see* **Note 3**). After 15 min rotation, collect 25µL of sample, place on a clean glass slide, cover the liquid drop with a coverslip and count clumps by fluorescent microscopy (**Fig. 1D**): acridine orange has an excitation/emission excitation and emission spectrum peak wavelengths of approximately 500nm/526nm when bound to DNA, and emits green fluorescence. A clump is defined as consisting of three or more infected erythrocytes and the frequency of the clumping phenotype in isolates is measured as the number of infected cells in clumps among 1,000 infected cells counted in duplicate assays. To assess clumping kinetics, take further samples at 30, 60, and 120 min, as described elsewhere [10,12].

2.2 Bridging

1. *Endothelial cells*. Grow endothelial cells to confluence on 12-well IFA slides kept inside sterile plastic Petri dishes (*see* **Note 4**). Stimulate half the wells with TNF (100 ng/mL in culture medium) overnight before the assay. Wash with pre-warmed PBS before use **(Fig. 2A)**.

2. *Blood samples.* Collect blood in vacutainers containing 3.8 M buffered sodium citrate as anticoagulant (ratio 1: 9).

3. *Purified platelet suspension preparation*. Pre-warm all the reagents at 37°C. Centrifuge blood samples immediately after collection at 250 *x g* for 10 min **(Fig. 2B)**. Carefully collect the cloudy yellow top PRP layer and further centrifuge for 10min at 2000 *x g* at room temperature or 22°C when using a temperature-controlled centrifuge **(Fig. 2C)**. Discard the PPP before proceeding to the platelet washing steps (*see* **Note 5**) **(Fig. 2D)**:
    a. Add 200 µL of Tyrode's buffer previously warmed at 37°C to the pellet before resuspending the cells very slowly and gently, being careful not to make bubbles. The pellet is small, so this is a tricky and crucial step.
    b. Once resuspended, or if you see small aggregates, add 1 mM of PGI_2, and gently mix to the cell suspension. When the platelets are resuspended and there aren't aggregates visible to the naked eye, top up to 10mL with warm Tyrode's buffer. The suspension should be cloudy and white.
    c. Stop the PGI_2 anti-aggreging action by incubating the suspension for 10 min at 37°C (incubator or water-bath), and centrifuge for 8 min at 2000 x g.
    d. Repeat (a) and (b) without adding PGI_2. In case of the presence of micro-aggregates, which is frequent, add 15 µl of heparin sodium solution to the 10mL before centrifugation and add a rinsing step to the protocol.
    e. Resuspend platelets in 1ml Tyrode's buffer, and adjust the density to $2x10^8$ platelets/mL using an hematocytometer (Malassez is preferred, but Neubauer works well too) by topping up the suspension with buffer.

4. *Parasitised red blood cells*. Cultivate a lab-adapted, CD36-binding *P. falciparum* strain, and select mature forms (trophozoites/schizonts) by gelatin flotation or MACS purification, as detailed in Chapter XX on general parasite culture **(Fig. 2E)**.

5. *Bridging assays.* Add platelets (platelet:endothelial cell ratio, 300:1) to endothelial cells and incubate for 90 min at 37°C. Rinse gently with warm PBS (5 times) and to remove nonadherent platelets, before adding PRBC (PRBC:endothelial cell ratio, 50:1) and further incubate the cell mixture for 90 min at 37°C. Rinse 5 times with warm PBS and to remove nonadherent PRBCs, and fix the remaining cells by immersion of the IFA slide in PLP overnight at 4°C in a transparent Petri dish. Count bound PRBCs in 10 randomly distributed fields the following day, by placing the Petri dish on an inverted microscope. The assay can be easily adapted to flow conditions (*see* **Note 6**).

Notes

1. PPP should not have more than 10 platelets/μL, whereas PRP should typically contains >300 $\times 10^3$ platelets/μL for healthy donors, and <150 $\times 10^3$ platelets/μL for CM patients
2. The use of Parafilm will not only ensure that the lid of the cryotube stays in place but will also allow the tube to get better traction on the roller.
3. This step is important to keep the cells in suspension through a gentle and steady rotation of the tube, and to increase the chance of contact between platelets and PRBCs. Any other equipment that can provide a slow, gently and constant movement of the tube (MACSmix tube rotator, etc.) can be used for the assays but the speed must be the same for each specific assay to allow unbiased comparisons.
4. The best IFA slides for this assay are the ones using a water-repellent coating to create convex “wells” by surface tension. The wells are smaller than the ones in 24-well plates, so the assays can be carried out using very small amounts of platelets/cells/reagents, which is particularly useful for bridging blocking assays with monoclonal antibodies. Two caveats need to be mentioned: first, because the slides are made of glass, most endothelial cell lines will require extracellular matrix coating before seeding. PBS with 1%

gelatin works very well, and the coating is done by filling the wells with the solution and incubating the slides for an hour at 37°C in a humidified chamber. Remove the PBS-containing gelatin immediately before seeding with endothelial cells, ensuring the wells stay wet between these steps. Second, because of the size of the wells, the culture medium evaporates faster than in plates with lids and the slides need to be kept in individual Petri dishes in the incubator before and during the assays. Monitor the level of medium in the wells and include a humidified chamber, if necessary.

5. Platelets are notoriously difficult to handle. They are activated by temperature drop and by motion, so the samples must be handled carefully and always kept at room temperature, especially for the bridging assays. Make sure all buffers and reagents are at 37°C to avoid aggregation due to temperature shock. When purifying platelets, resuspend them very gently. The pellet can take a while to disaggregate, don't rush the process. Even when all adequate measures are taken, platelets can aggregate at any stage of the purification. It is therefore advisable to purify them by batches to have a back-up source in case that happens.
6. For flow assays, endothelial cells can be seeded and cultured to confluence in Ibidi chambers, for example, or Flaskettes that can be attached to a separate plexiglass flow chamber. In that case, the platelet suspension should be allowed to flow on endothelial cells stimulated with TNF overnight for 10min at a maximum pressure of 0.05 Pa to avoid activating platelets before rinsing with PBS for 15 min at 0.05 Pa. PRBC suspensions should then be allowed to flow through the chamber for 10min at 0.05 Pa, followed by fresh RPMI 1640 to remove nonadherent cells. As for static conditions, flood the chamber with PLP and fix overnight at 4°C before counting adherent PRBC in 10 randomly distributed fields

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Figure Captions

Figure 1: **Step-by-step protocol for in vitro bridging assays.** Step 1: grow endothelial cells on IFA slides and stimulate with TNF overnight before the assay (A). Step 2: on the day of the assay, centrifuge freshly collected whole blood to separate the PRP (B), centrifuge further to pellet platelets (C) and wash them to generate a suspension in buffer (D). Step 3: prepare a suspension of mature forms of PRBC (E). Step 4: add platelets followed by PRBC to endothelial cells (F) to assess the in vitro bridging (G).

Figure 2: **Step-by-step protocol for ex vivo clumping assays.** Step 1: centrifuge freshly collected whole blood from patients or controls to pellet PRBC (A). Step 2: collect and store the PRP (B). Step 3: culture clinical isolates for up to 48h (C). Step 3: add the PRP (or PPP for controls) to cultured PRBC to assess the ex vivo clumping (D).

Table Captions

Table 1: Reagents and concentrations needed to prepare Tyrode's buffer. The final pH needs to be adjusted to 6.9.

Table 2: Reagents and concentrations needed to prepare paraformaldehyde-lysin-periodate (PLP). The final pH needs to be adjusted to 7.4

Tables

Table 1

Reagent	Final concentration
NaCl	0.137 mol/L
KCl	3 mmol/L
NaH_2PO_4	0.4 mol/L
$NaHCO_3$	12 mmol/L
$MgCl_2$	1 mmol/L
HEPES	14.7 mmol/L
Glucose	20 mmol/L
Bovine serum albumin	0.2%

Table 2

Reagent	Final concentration
NaH_2PO_4	37 mmol/L
Na_2HPO_4 7H2O	37 mmol/L
NaIO4	1 mmol/L
DL-lysin-HCl	76 mmol/L
Paraformaldehyde	20%