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Evaluation of Pregnancy Malaria Vaccine Candidates: The Binding Inhibition Assay

Tracy Saveria, Patrick E. Duffy, and Michal Fried

Abstract

The parasite-binding inhibition assay is designed to evaluate the acquisition of naturally acquired functional antibodies that block *Plasmodium falciparum* binding to endothelial or placental receptors. The assay is also used to assess functional activity by antibodies induced by immunization, for example antibodies raised against pregnancy malaria vaccine candidates like VAR2CSA. Here we describe a plate-based assay to measure the levels of adhesion-blocking antibodies. This assay format can be adapted to any lab that is minimally equipped for short-term parasite culture.

Keywords

Binding inhibition assay; Plate-based assay; IgG purification; CSA

1 Introduction

During pregnancy, *Plasmodium falciparum* parasites sequester in the placenta and adhere to chondroitin sulfate A (CSA) expressed on the surface of the syncytiotrophoblast [1]. Malaria during pregnancy is a major public health problem, associated with severe maternal anemia as well as with low birth weight (LBW) delivery and infant mortality [2, 3]. In areas of stable malaria transmission, the hallmark of pregnancy malaria is parity-dependent susceptibility, whereby women develop resistance over 1–2 pregnancies that controls infection and prevents severe sequelae. This unique epidemiology results from acquisition of immunity to placental parasites, and therefore a vaccine to prevent malaria during pregnancy should mimic naturally acquired immunity acquired by multigravid women.

Currently, an international effort to develop a vaccine to prevent pregnancy malaria focuses on a member of the PfEMP1 variant surface antigen family named VAR2CSA. VAR2CSA is preferentially expressed by placental parasites and parasite isolates selected for binding to CSA [4], and women acquire antibodies to VAR2CSA over successive pregnancies as they develop resistance to placental malaria. VAR2CSA is a large protein of about 350 kDa composed of six extracellular Duffy binding-like (DBL) domains, and is too large to manufacture as an intact molecule. Therefore immunogens are being considered that incorporate one or a combination of VAR2CSA's DBL domains, with or without adjacent interdomain regions.

One of the major challenges in developing a pregnancy malaria vaccine (PMV) is identifying a domain or domain combination that can elicit broadly reactive antibodies. In endemic areas women acquire strain-transcending antibodies that block parasite adhesion to CSA and this property has been an important criterion in selecting candidates. A number of studies have evaluated PMV candidates by assessing the level of inhibition of adhesion by antibodies raised to recombinant forms of VAR2CSA domains or full-length protein.

Several platforms for binding inhibition assays have been described, including a high-throughput assay in 96-well plates of tritium-labeled parasites [5] and a flow-based assay [6]. Here, we focus on the static Petri dish-based assay [7]. This assay format can be adapted to any lab that is minimally equipped for short-term parasite culture, a centrifuge, and a microscope. Therefore, the assay can be used and has been used to analyze anti-adhesion activity on fresh parasite isolates that can thus support the selection of the optimal PMV candidate.

2 Materials

2.1 Reagents

1. Trophozoite-stage parasites at 5–20 % parasitemia, 0.5 % hematocrit (*see* Subheading 3.1).
2. 100 mm × 20 mm polystyrene Petri dishes (Falcon 351029) (*see* Note 1).
3. Sterile phosphate-buffered saline (PBS), pH 7.4.
4. Sterile RPMI1640 media.
5. 1 % Gelatin (Sigma G2625) in sterile RPMI 1640 media.
6. Bovine serum albumin (BSA), powder (Sigma A5611 or similar product from other manufacturers).
7. 3 % BSA in sterile PBS.
8. 3 % BSA in sterile RPMI 1640 media.
9. 1.5 % Glutaraldehyde (Sigma G6257) in sterile 1× PBS.
10. 5 % Giemsa (Sigma GS-500) in deionized water or 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma D9542) in deionized water.
11. Chondroitin sulfate A (CSA), sodium salt, from bovine trachea (Sigma C9819-25G).
12. Control and test sera and/or IgG (*see* Subheading 3.3).

2.2 Equipment

1. Tabletop centrifuge (that allows for centrifugation at 1800 × *g*).

¹The type of Petri dish plate is critical. Falcon 351029 Petri dishes were found to be suitable. Any other plate should be compared to this plate to determine if the plate is suitable for the assay.

2. Microcentrifuge.
3. Laminar flow hood.
4. Incubator at 37 °C.
5. Water bath at 37 °C.
6. Vacuum pump.
7. Rotator (optional).
8. Peristaltic pump (optional).
9. Light or fluorescent microscope.

3 Methods

The binding inhibition assay for CSA-adherent parasites can be used for immuno-epidemiological studies to evaluate acquisition of immunity in exposed populations using human plasma, sera, or IgG. The assay can also evaluate functional activity in animal anti-sera raised against PMV candidates. For both applications, confirmation of parasite binding to CSA is performed prior to the analysis of anti-adhesion activity to ensure that the parasites are suitable for these measurements.

3.1 Parasite Preparation

Parasite isolates can be from one of the following sources:

- 1 Laboratory-adapted parasite lines selected for binding to CSA, such as *P. falciparum* CS2 available from MR4 (MRA-96).
- 2 Fresh isolates obtained from the peripheral blood of pregnant women (*see* Note 2).
- 3 Blood collected from infected placentas (*see* Note 2).

Parasites collected from the peripheral blood are commonly at the ring stage and should be allowed to mature in in vitro culture for about 12–20 h until reaching trophozoite/schizont stages, which adhere to endothelial receptors. Placental blood commonly contains mature parasite forms that can be used immediately in the assay. To evaluate anti-adhesion activity, parasites are enriched to a parasitemia of >1 % using a gelatin gradient.

Gelatin enrichment of mature-form parasites:

- 4 Warm the 1 % gelatin solution in a 37 °C water bath.
- 5 Pellet the blood at $500 \times g$ for 5 min and resuspend with RPMI 1640 to 50 % hematocrit.
- 6 Add two volumes of gelatin solution and incubate for 30 min in a 37 °C water bath. In the absence of a water bath, an incubator set at 37 °C can be used.

²Informed consent should be obtained from donors before collecting blood or placenta.

- 7 Mature parasites remain in the upper layer while uninfected erythrocytes and ring-stage parasites form rouleaux and descend to the bottom layer.
- 8 Transfer the upper layer to a new tube, pellet the blood containing mature parasites, and wash three times with RPMI 1640 media.
- 9 Prepare and read a thin blood smear using methanol fixation followed by Giemsa staining: if parasitemia is >20 %, dilute to 20 % with uninfected red blood cells.

3.2 Plate Preparation

1. Using a template, draw circles on the back of a polystyrene Petri dish and label the wells appropriately. To prepare a template, use a circular Petri dish-sized piece of material (e.g., plastic, cardboard, paper) that contains twenty 10 mm circles drawn in 18° increments around the perimeter. Each circle will be able to accommodate approximately 20 µl of liquid (*see Note 3*).
2. Apply 15–20 µl of CSA solution at a concentration of 20 µg/ml (in sterile PBS) to the interior surface of the Petri dish within the margins of the marked wells.
3. Allow the CSA to absorb overnight at 4 °C in a humid chamber (e.g., a sealable container with wet paper towels).
4. Alternatively, plates can also be prepared on the same day by incubating for 4 h at 37 °C in a humid chamber.

3.3 IgG Purification of Human and Animal Sera

The method described here is based on using Protein G-coupled GammaBind Sepharose beads (GE Healthcare). Alternatives are available at your own discretion.

3.3.1 Materials

1. IgG Binding Buffer (Thermo Scientific 21019).
2. IgG Elution Buffer (Thermo Scientific 21004).
3. Neutralization buffer (1 M Tris–HCl pH 8.5).
4. Dialysis material for 10 K MWCO (suggest using Thermo Scientific Dialysis Cassettes, 10 K MWCO; requires needles/syringes for application).
5. Centrifuge columns, 0.8 ml (e.g., Pierce PN89868).
6. GammaBind™ Plus Sepharose™ bead slurry (GE Healthcare 17-0886-01).

3.3.2 Procedure

1. Bring all buffers to room temperature.

³Throughout the procedure, it is very important to prevent drying of spots. Apply blocking solution or parasite suspension immediately after aspirating liquid from each well.

2. Add 400 μ l of bead slurry to the centrifuge column (gently swirl slurry before application to ensure homogenous suspension); twist bottom off column and place in 1.5 ml microcentrifuge tube.
3. Spin for 1 min at $5000 \times g$ (all spins are performed for the same time and force).
4. Wash column/beads using 400 μ l binding buffer; spin and discard flow through. Repeat one more time.
5. Apply the centrifuge column cork to the bottom of the column.
6. Add 100–450 μ l of plasma/serum sample on top of the beads (optimal volume is 150 μ l); cap the column (Parafilm[®] wrap is recommended to prevent leakage).
7. Incubate with gentle rotation end over end, for 20–30 min at 4 °C.
8. Remove cork from column and spin; discard flow through (this can be saved to determine binding efficiency).
9. Wash using 400 μ l of binding buffer, inverting the column several times after buffer application. Discard flow through after spin. Repeat wash step twice.
10. Add 25 μ l of neutralization buffer to a clean microcentrifuge tube; transfer column to this tube.
11. Add 400 μ l of elution buffer to column.
12. Spin; keep flow through containing purified IgG.
13. Repeat **steps 10 – 12** twice more, using the same column but with fresh microcentrifuge tubes, neutralization buffer and elution buffer.
14. Combine the IgG obtained in the three elutions. Samples are dried prior to dialysis by either lyophilization/freeze-dry or evaporation. Punch a hole on the top of each tube; freeze on dry ice before adding to lyophilizer (run on lyophilizer overnight if necessary) or alternatively, samples can be dried using a SpeedVac[™] concentrator.
15. Add 100 μ l of water to each tube of dried sample; if samples are aliquoted into multiple tubes combine into one tube.
16. Dialyze against 1 l of 1 $\times$ PBS for 30–60 min at RT. If using recommended dialysis cassettes, be sure to hydrate the cassette in PBS for 1 min before application.
17. Carefully add sample using needle and syringe; remove all air from the cassette.
18. Float cassette in PBS.
19. Use a magnetic stir bar for gentle stirring of the PBS dialysis medium.
20. Change to fresh 1 l 1 $\times$ PBS and dialyze for 1–2 h at 4 °C, again with gentle stirring.
21. Change to fresh 1 l of PBS and dialyze overnight at 4 °C, again with gentle stirring (can add an additional PBS change if preparing multiple samples).

22. Remove sample from dialysis membrane and place in fresh microcentrifuge tube.
23. If using dialysis cassette, use needle and syringe to put a small amount of air into cassette before removing sample.
24. Keep sample at 4 °C if planning to use within 48 h; otherwise aliquot and store at −20 °C.
25. Determine the IgG concentration using the BCA assay (Pierce 23225) or similar methodology of your choosing.

3.4 Binding Inhibition Assay: Setup

Binding inhibition assays can be used to test plasma, serum, or IgG from malaria-exposed humans or from animals immunized with vaccine candidates (*see* Note 4). These samples are defined as “test sera.” The level of binding is compared to the level of binding in the presence of nonimmune serum/plasma or serum/plasma from animals immunized with an irrelevant protein that is expressed and formulated in a similar way as the vaccine candidate (*see* Note 5). Inhibition assays are also performed using purified IgG (*see* Subheading 3.3).

1. Incubate parasites at a parasitemia of >1 % and hematocrit of 0.5 % in 3 % BSA/RPMI 1640 media for 30 min at 37 °C or room temperature.
2. Appropriately label microcentrifuge tubes with nomenclature for the serum or IgG being tested. Gently mix the parasite suspension to ensure even distribution and then divide among the labeled microcentrifuge tubes, adding 15–20 µl per tube (use the same volume as the initial receptor volume throughout the assay).
3. Spin for 2 min at 400 × *g* and remove the volume of supernatant equal to the volume of serum to be added. Human serum or plasma is commonly used at a dilution of 1:5–1:10. The appropriate dilution of sera raised against recombinant proteins should be determined.
4. Purified IgG is analyzed at increasing concentrations (0.1 mg/ml thru 1 mg/ml). To maintain an equal concentration of BSA among the samples tested with varying amounts of IgG, remove the volume of supernatant equal to the volume of the highest IgG concentration to be used from all the tubes. Add increasing amounts of IgG and RPMI 1640 media to a final volume of 15–20 µl.
5. Make sure that the parasites are well mixed with the serum/IgG to ensure proper binding of the antibodies to the parasites.
6. Incubate the parasites in serum/IgG for 30 min at 37 °C or room temperature.
7. Block the CSA plates with 3 % BSA/PBS: aspirate the CSA spot and immediately apply 15–20 µl of 3 % BSA/PBS; incubate for at least 30 min at

⁴When using human plasma in the inhibition assay, plasma blood type must be matched to parasite blood type to avoid agglutination. It is not necessary to match blood type for long-term cultured parasites adapted to blood type O.

⁵Inhibition assays should always include controls. Assays performed with human plasma (or IgG) should include nonimmune sera (e.g., sera collected from malaria-naïve donors) as a negative control, and pooled immune sera from multi-gravid women as a positive control. Assays of sera (or IgG) from immunized animals should include a negative control from animals that were immunized with an irrelevant protein produced from the same expression platform and prepared and delivered as immunogen in the same way as the test reagents.

room temperature in a humidified container (e.g., sealable container with moist paper towel, or humidified incubator).

8. Aspirate the BSA/PBS blocking buffer from the wells by suction and immediately add the parasite suspension from step 6. Incubate for 30 min at room temperature in humidified container.

3.5 Binding Inhibition Assay: Washes

3.5.1 Manual Washing

- 1 To wash the plate, cover the samples with 20 ml of PBS by slowly adding PBS to the center of the Petri dish (*see* Note 6).
- 2 Avoid adding PBS directly to the parasite suspensions.
- 3 After the wells are covered with PBS, tilt the plate gently to wash off unbound cells. Collect PBS and dislodged cells by suction at the edge of the dish.
- 4 Repeat the washing process three times.

3.5.2 Automated Washing Station—An automated washing station was designed to allow consistent plate washing to reduce the variation between plates. The system was previously described [8] and includes a rotating platform and a peristaltic pump that regulates the inflow and outflow of the washing solution. The peristaltic pump delivers washing buffer (PBS) through the inlet tubing until the buffer reaches the level of the outlet tubing.

- 5 Wash in PBS on a rotating platform, by slowly and carefully adding PBS to the center of the plate, and allowing it to gently flow and fill the plate (*see* Note 6). When necessary, gently vacuum extra liquid and blood from the center of the plate (*see* Note 6). Continue to gently wash until the spots are clear of blood and there is no longer any blood concentrated in the center of the plate.
- 6 Discontinue PBS addition. Aspirate the remaining PBS using vacuum while the rotating platform is still on—be sure to aspirate in the middle of the plate first, only aspirating from the edges when collecting the final PBS.

3.6 Binding Inhibition Assay: Fixing and Staining

1. Add 10 ml of 1.5 % glutaraldehyde/PBS to the Petri dish and incubate for 10 min at RT.
2. Gently rinse the plate three times with deionized water (gently adding to the center of the plate, not on the spots).
3. Stain with 5 % Giemsa for 5 min or 1 µg/ml DAPI in PBS for 10 min (if using DAPI, cover plates after final rinse and keep away from light).
4. Gently rinse plate three times with deionized water and let the plates dry.

⁶Be careful to avoid aspirating parasites directly from wells.

3.7 Binding Inhibition Assay: Counting

3.7.1 Giemsa-Stained Plates

- 1 Count bound infected red blood cells (iRBCs) stained with Giemsa using a 10× eyepiece and 100× magnifying objective (high-power field). Survey each test site to identify areas with low background (no/few uninfected RBCs).
- 2 Count 20 fields that contain the highest number of parasites.
- 3 Follow the same approach (survey for low-background areas and count fields with highest parasite densities) for all the wells.
- 4 To calculate the level of inhibition, compare the number of bound cells in the presence of test sera (N_{test}) to the number bound in the presence of control sera (N_{control}). Calculate the percent inhibition: $100 - ((N_{\text{test}}/N_{\text{control}}) \times 100)$.

3.7.2 DAPI-Stained Plates

- 5 Use MetaMorph® or other standard imaging software. Count a minimum of 1000 red blood cells (over several fields of view), using a 10× eyepiece and 40× magnifying objective fluorescent microscope with a UV laser in order to determine the number of DAPI-stained cells, noting both the number of infected and uninfected cells.
- 6 To calculate the level of inhibition, compare the number of bound cells in the presence of test sera (N_{test}) to the number bound in the presence of control sera (N_{control}). Calculate the percent inhibition $100 - ((N_{\text{test}}/N_{\text{control}}) \times 100)$.

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