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Measurement of Antibody-Mediated Reduction of *Plasmodium yoelii* Liver Burden by Bioluminescent Imaging

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Abstract

Antibodies against the infectious sporozoite stage of malaria have been shown to be effective in preventing infection of the liver and in mitigating the ensuing blood stage. However, only a handful of antibody targets have been vetted and shown to be successful in mediating in vivo protection. Even more limited are the means with which to measure how effectively antibodies can reduce the number of parasites establishing infection in the liver. Traditionally, only qPCR of infected mouse livers could accurately measure liver parasite burden. However, this procedure requires sacrifice of the animal and precludes monitoring of the ensuing blood stage infection. Herein we describe a method of accurately assessing antibody-mediated reduction of parasite liver burden by combining passive or active immunization of mice and mosquito bite challenge with luciferase-expressing transgenic *P. yoelii* parasites. This method is rapid, reliable and allows for observation of blood stage disease in the same animal. This model will prove integral in screening the efficacy of novel antibody targets as the search for a more effective malaria vaccine continues.

Keywords

Antibody; Humoral immunity; Malaria; Liver stage; Pre-erythrocytic; Bioluminescent imaging

1 Introduction

Historically, the majority of antibody (Ab)-based experimental vaccines for malaria control have been focused on the disease-causing blood stage parasite. As a result, the role of Abs in mediating protection at this stage has been well established and a number of stage-specific assays are available to measure the effects of Abs both in vitro and in vivo [1–4]. However, the pre-erythrocytic (skin to liver) stage of the parasite is an extremely attractive target for vaccine intervention given the low number of parasites at this stage and the lack of clinical symptoms during this stage. Data from clinical trials using live-attenuated sporozoites or a pre-erythrocytic subunit vaccine (RTS,S) have stoked this interest as they both appear to rely heavily on anti-sporozoite Abs in mediating protection [5–9]. The RTS,S vaccine targets the major sporozoite surface protein, circumsporozoite protein (CSP), and this remains one of only a few sporozoite Ab targets identified to date. As the search for additional Ab targets intensifies, there will be a need to evaluate the ability of Abs to prevent the establishment of a parasite liver stage infection and subsequent blood stage infection in an efficient and physiologically relevant manner.

Currently, mouse models of malaria allow for noninvasive and accurate measurement of blood stage disease through either Giemsa-stained blood smears or flow cytometry [1, 2, 10–14]. Both of these methods, in addition to in vitro growth assays for *P. falciparum*, have allowed for extensive pre-clinical analysis of Ab-based interventions for blood stage malaria [15]. Prior to in vivo imaging, the only way to quantify parasite liver stage burden has been through labor-intensive counting of liver stage parasites via microscopy or by measuring liver parasite RNA using quantitative real-time PCR. While accurate, both of these methods require sacrifice of the animal, which precludes evaluation of the subsequent blood stage infection. Here, we describe an efficient and noninvasive method of assessing Ab-mediated inhibition of liver stage infection by challenging passively or actively immunized animals with a transgenic *P. yoelii* parasite that expresses a luciferase reporter [16]. This method is technically simple, accurate and does not require sacrifice of the animal—allowing for time course analysis of liver infection and for monitoring the ensuing blood stage infection.

1.1 Basic Experimental Design

Several bioluminescent malaria parasites exist for both *P. berghei* and the *P. yoelii* YM (lethal) strain [17–20]. However, the methods described here are optimized for a transgenic non-lethal *P. yoelii* XNL strain that expresses a GFP-luciferase fusion (Py-GFP-luciferase) [16]. Aside from non-lethality, this transgenic parasite has the advantage of being detectable at earlier time points of liver stage infection compared to similar transgenic parasites [17–20]. The parasite was generated by integrating a GFP-luciferase fusion-expressing construct into the dispensable *S1* locus [16]. The parasite develops normally and expresses luciferase throughout all stages of parasite development in both the mosquito and mouse [16]. Following intravenous injection of 10^5 sporozoites, parasite liver infection can be detected by in vivo bioluminescent imaging as early as 16 h with a peak between 42 and 48 h as the parasites begin the transition from the liver stage to the blood stage [16] (Fig. 1). Bioluminescent quantification of liver burden using this parasite has been shown to be as accurate as qPCR although not quite as sensitive at very low levels of infection. Thus, this system allows for a more efficient and immediate assessment of liver burden while enabling continued survival of the animal for monitoring of blood stage disease.

Here, we describe how this method can be combined with active or passive immunization of animals to assess Ab-mediated inhibition of liver infection in mice. It is important to note that although inhibition of liver infection can be demonstrated with passive transfer of large doses of monoclonal Ab (mAb) followed immediately by intravenous sporozoite injection, detection of Ab-mediated reduction in liver infection is most sensitive when the challenge is performed via mosquito bite at least 16 h after passive transfer of Ab [21–24]. Using this methodology, it is possible to detect a wide range of inhibition of liver infection with subsequent follow up of blood stage infection (Fig. 1).

Passive transfer of mAb followed by mosquito bite infection and bioluminescent imaging has been rigorously verified as an effective means to detect Ab-mediated inhibition of liver infection [25]. Evaluation of Abs from other sources such as purified polyclonal Abs or serum containing polyclonal Abs against a particular target is also possible. Furthermore, the ability of serum containing Abs to multiple antigens (e.g., from mice immunized with live

attenuated parasites) to inhibit liver infection has also been evaluated by this method [26]. However, passive transfer of serum substantially dilutes the Ab concentration and is limited by the amount that can be safely injected into a mouse. Thus, serum for passive transfer may not be available in sufficient quantities to mediate an observable effect especially in the case of serum from single antigen immunizations. In this case, it is preferable to actively immunize mice with a protein/peptide in adjuvant to elicit high-titer Abs *in vivo*. These immunized mice can then be depleted of CD4⁺ and CD8⁺ cells immediately prior to challenge to eliminate the possibility of T cells inhibiting liver infection. All of these methods are described in detail below.

2 Materials

2.1 Preparation of Immune Sera and Plasma

1. Attenuated live sporozoites or purified antigen in adjuvant (*see* Subheading 2.3).
2. 250 IU/mL heparin in phosphate buffered saline (PBS), pH 7.4.
3. 30 gauge needles.
4. 1 mL syringe.
5. Serum separator collection tubes (BD Microtainer, cat# 365956).
6. 1.5 mL microcentrifuge tubes.
7. Tabletop centrifuge.

2.2 Passive Transfer of Immune Sera/Ab

1. Immune sera or purified Ab (without sodium azide).
2. Purified antigen.
3. Adjuvant of choice.
4. Syringe appropriate for adjuvant method.
5. 27 gauge needles.
6. 1 mL 30 gauge insulin syringes.
7. 1.5 mL microcentrifuge tubes.
8. PBS.
9. Heat lamp.
10. Ethanol pads.
11. BALB/cJ mice.

2.3 Direct Challenge of Mice Actively Immunized with Peptide/Protein

1. Purified antigen in PBS.
2. Adjuvant of choice.

3. Syringe appropriate for adjuvant method.
4. 1 mL 30 gauge insulin syringes.
5. Anti-CD4⁺ mAb (clone GK1.5).
6. Anti-CD8⁺ mAb (clone 2.43).
7. PBS.

2.4 Py-GFP-luc Mosquito Bite Infection

1. Small cages suitable for mosquito containment.
2. Mouth aspirator (John W. Hock Company, model 612).
3. Day 14 Py-GFP-luc infected mosquitoes.
4. Xylazine.
5. Ketamine.

2.5 Bioluminescence Imaging

1. RediJect D-Luciferin (Perkin Elmer product # 760504, or equivalent).
2. 30 gauge needles.
3. 1 mL 30 gauge insulin syringes.
4. Black construction paper.
5. Isoflurane.
6. Isoflurane anesthesia system.
7. IVIS Lumina I animal imager (or equivalent).
8. Computer with Living Image software or equivalent.

3 Methods

3.1 Preparation of Immune Sera for Passive Transfer

1. Immunize 10 BALB/c] mice with 2–3 doses of attenuated parasites or 3 doses of your antigen of choice in adjuvant at 2–4 week intervals (*see* Note 1). Mock immunize 10 mice as a control either with uninfected mosquito salivary gland extract or with an irrelevant protein in adjuvant.
2. Nine to fourteen days following the last immunization, euthanize the immunized mice by CO₂ asphyxiation and collect the mouse blood by cardiac puncture using a 27-gauge needle on a 1 mL syringe. Do not heparinize the syringe if serum (as opposed to plasma) is desired. Transfer the blood to a 1.5 mL microcentrifuge tube. Alternatively, serum separator tubes containing a gel

¹All animal experimentations must be approved by the relevant committees (for example, in the USA, such work is approved by the IACUC—the Institutional Animal Care and Use Committee) and personnel performing procedures on animals must receive appropriate training.

matrix to separate cells from serum can be used (BD Microtainer tubes). In this case, transfer the blood immediately to these tubes and follow manufacturer's instructions for serum collection.

3. If plasma is preferred, coat the syringe with heparin by drawing 200 μL of heparin solution into the syringe and fully extending the plunger so the heparin comes into contact with the entire length of the syringe. Next, ensure that the heparin is in contact with the rubber of the plunger by holding the syringe vertical and tapping it until the all bubbles rise to the top. Fully depress the plunger, expelling excess air and heparin, leaving a small amount of heparin in the dead space between the plunger and needle.
4. Allow blood to clot for 30 min at room temperature. If collecting plasma, proceed directly to **step 6**.
5. Centrifuge clotted blood at $10,000\times g$ in a tabletop centrifuge for 10 min to separate serum.
6. The serum/plasma will separate as a supernatant above pelleted cells. Collect and pool (optional) serum from the 10 immunized mice. Store at $-20\text{ }^{\circ}\text{C}$ until use.

3.2 Passive Transfer of Immune Sera/Ab

1. If analyzing purified Ab, dilute Ab in PBS to desired concentration. If the Ab preparation contains sodium azide, this must be removed by standard dialysis/de-salting procedures. Do not dilute immune sera.
2. Load 1 mL insulin syringes with 30 gauge needles with 300 μL of undiluted immune sera or desired amount of diluted Ab (to a total volume of up to 300 μL). A good positive control should be included (*see Note 2*). Total Ab mass should be less than 500 μg .
3. A mock injection of species-matched (isotype-matched optional) Ab in a quantity matching the highest dose should also be administered. For serum passive transfer, an equal volume of serum from mock-immunized mice should be administered.
4. Inject the Ab/sera into the mice via the tail vein. To ease the injection process, the mouse should be pre-warmed with a heat lamp placed over a cage containing the mouse. The mouse is ready for injection when it begins to groom more often—a sign that the mouse is warm, making the tail vein dilated and easier to locate/inject. Wiping the mouse tail with an ethanol pad prior to injection will also bring the tail vein to the surface, enhance visualization of the vein, and ease injection.
5. Wait 16–24 h before proceeding to the challenge step (*see Note 3*).

²One example is a 150 μg /mouse dose of the anti-CSP repeat mAb (clone 2 F6) which is enough to reduce liver burden to background levels in BALB/cJ mice and prevent development of blood stage in ~50 % of mice.

³This allows for distribution of the antibody throughout the mouse tissue and greatly enhances the ability of antibodies to function in the skin. Failure to wait will result in decreased sensitivity of antibody activity [25].

3.3 Active Immunization and Challenge of Immunized Mice

1. Immunize at least 5 BALB/cJ mice per group with antigen in adjuvant or live attenuated parasites according to Subheading 3.1. Immunize an equal number of mice with an irrelevant antigen in adjuvant or mosquito salivary gland extract respectively.
2. At least 3 weeks following the final immunization, mice are ready to be challenged. Prior to challenge, it is wise to confirm the presence of serum Abs against the parasite or antigen by collecting a small amount of blood and analyzing via ELISA.
3. To deplete mice of T cells, prepare a solution of 0.5 mg/mouse of anti-CD4⁺ (clone GK1.5) and 0.35 mg/mouse anti-CD8⁺ (clone 2.43) Abs in a total of 200 μ L of PBS per mouse.
4. Using a 1 mL syringe with a 30-gauge needle, inject 200 μ L of the Ab cocktail intraperitoneally into each mouse.
5. Beginning at 6–12 h after administration of T cell depleting Abs, depletion can be confirmed by analyzing peripheral blood cells for the presence of CD4⁺ and CD8⁺ T cells using standard flow cytometry procedures.
6. Mice are ready for challenge 16–24 h after T cell depletion.

3.4 Py-GFP-luc Mosquito Bite Infection

1. Infect *Anopheles stephensi* mosquitoes with Py-GFP-luc gametocytes via an infectious blood meal using standard procedures [27].
2. On day 10 after mosquito infection, quantify mosquito infection rate by analyzing 10–12 mosquito midguts for the presence of oocysts (*see* Note 4).
3. On day 14 post mosquito infection, quantify mosquito salivary gland infection by dissecting 10–20 mosquitoes using standard procedures (*see* Note 5) [27].
4. Using a mouth pipette, transfer 15–20 mosquitoes into small containers modified with netting for housing mosquitoes, one per mouse to be infected (Fig. 2). Mosquitoes from different cages/infections can have varying levels of infection rates and salivary gland sporozoite numbers. If using mosquitoes from more than one starting cage, first transfer all mosquitoes into one cage and then aliquot into the bite cages to allow for equal distribution of mosquitoes, which may have varying infection rates between individual cages.
5. To improve the likelihood of mosquito feeding, starve mosquitoes 3–6 h prior to infection by removing the sugar pad or replacing the sugar pad with a pad soaked only in water.

⁴.In general, it is recommended to use mosquitoes from a cage that have an infection rate >50 % with the majority of mosquitoes harboring >10 oocysts per midgut.

⁵.Only use mosquitoes with an average of >10,000 sporozoites/mosquito for mosquito bite infection.

6. Immediately prior to mosquito bite infection, mice must be anesthetized. To do this, the mice are injected intraperitoneally with 8 μ L/g body weight with a diluted Ketamine/Xylazine solution (1 mL Xylazine [20 mg/mL] + 2 mL Ketamine [100 mg/mL] + 13 mL sterile saline PBS) with a 1 mL insulin syringe.
7. Once mice are anesthetized (mice will be motionless, eyes open, slow but controlled breathing), place mice on small cages containing 15–20 mosquitoes, one mouse per cage. Begin timing infection and after 1 min, briefly lift each mouse from the top of the cage and replace to encourage mosquito bite probing (when sporozoites are injected) rather than feeding. After another minute (2 min after start of infection) rotate mice between different cages. This will minimize variation in mouse infection due to any cage-to-cage variation in mosquito bite infection. Repeat this lifting /rotating process for 10 min total. After 10 min, allow the mice to recover in a warm and humid environment to regain mobility before being returned to their cages.

3.5 Bioluminescent Imaging of Liver Stage Burden

1. Differences in liver stage burden are best assessed at the peak of liver infection, which is between 42–48 h for *P. yoelii* liver stage development. Thus, begin preparing for in vivo imaging during this time frame.
2. Thaw vials of RediJect D-luciferin (or equivalent luciferin) at room temperature in the dark in sufficient quantities to inject 100 μ L/mouse.
3. If using mice with pigmented fur, it is necessary to shave the abdomen of the mouse with clippers in order to prevent absorption of light signal from the liver. It is necessary to shave the abdomen liberally, from the hip line to just above the rib cage vertically and mid-way to the back on each side.
4. Using a 1 mL syringe with a 30-gauge needle, intraperitoneally inject mice with 100 μ L (or according to manufacturer's instructions) of luciferin 5–10 min prior to imaging. Bioluminescent output is constant between 5 and 20 min after injection, which allows for time to anesthetize and image mice. At this point, it is advised to inject mice in groups according to the number of mice to be imaged at once (typically 1–5, depending on imaging setup).
5. A piece of black construction paper can be used on the imaging surface for ease of cleaning following mouse imaging.
6. 2–3 min after luciferin injection, anesthetize mice using isoflurane in a box chamber at a flow rate according to the manufacturer's recommendations. Once mice are fully anesthetized, transfer to the imaging chamber of the bioluminescent imager.
7. Exact settings for each machine will vary, but using Living Image Software v3.0 with a Caliper Life Sciences in vivo imaging system (IVIS), consistent results can be obtained by acquiring images with a 10-cm-diameter field of view (FOV), a medium binning factor, and an exposure time of 2 min. Liver stage burden in

control mice (infected following administration of mock Abs) should be visible at the anatomical site of the liver (see Note 6).

8. Immediately following imaging, return mice to their cage and monitor for quick recovery from isoflurane anesthesia.
9. Save the image of the mice according to software manufacturer's instruction.
10. Mice may now be monitored for effects on patency/parasitemia on days 3–21 via Giemsa-stained thin smear or flow cytometry using standard techniques.

3.6 Analysis of Liver Stage Burden

1. Quantitative analysis of bioluminescence can be performed by measuring the luminescence signal intensity utilizing the region of interest (ROI) settings of Living Image 3.0 software (XGI-8; Caliper Life Sciences). To measure the liver burden of each mouse, place ROIs of equal size (using copy and paste) around the area at the location of the liver for each mouse. Ensure each ROI within each image and between images/groups to be compared are of the same exact size. Liver burden is directly correlated with total flux values (in photons/second [p/s]) for each ROI and this value should be used for all comparisons of liver stage infection. Ensure the ROI's are measuring "radiance" which will display the total flux above each ROI.
2. Background flux can be determined by placing ROIs over the pelvis of each mouse where luminescence should be absent at 42–48 h post-infection. An example of ROI use for measuring liver burden and background can be seen in Fig. 3.

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⁶If there is no or low burden detectable, the exposure time can be increased to 5 min. Alternatively, the mice in question can be re-injected with luciferin to ensure there was not a problem with the luciferin injection.

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Fig. 1. Antibody-mediated inhibition following mosquito bite sporozoite challenge. Representative images (**a**) and quantification of liver burden (**b**) are shown from mice that were administered control antibody (“mlgG”) or indicated doses of the anti-*P. yoelii* CSP monoclonal antibody (mAb) 2F6 prior to challenge by bite of 15 mosquitoes infected with *P. yoelii* expressing GFP-luciferase

Fig. 2.
Infected mosquitoes set up in small cages with a netting barrier to allow for a mosquito bite infection

Fig. 3.
Example of region of interest (ROI) placement for measuring liver stage burden and background luminescence