2-cycle GIA with *p*LDH assay

Created by: B.P.R.C. Date: November 2010

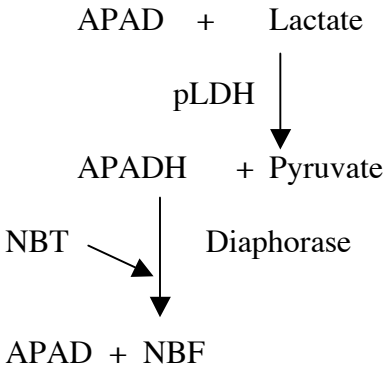
Aim

The aim of this 2-cycle Growth Inhibition Assay is to quantify inhibition of invasion or growth of *P. falciparum* into erythrocytes. The inhibition is thought to be mediated by P. falciparum specific antibody in the presence of purified IgG fraction from animals immunized with *P. falciparum* antigens. Allowing the parasites to grow for two cycle facilitates the detection of the biological effects of antibodies that act later in the life cycle (growth inhibition rather than invasion inhibition). The read out system is detection of *p*LDH (*plasmodium* Lactate DeHydrogenase), this enzyme is produced by live parasites and well detectable when they are in late trophozoiet and schizont phase.

Principle

3-Acetylpyridine Adenine Dinucleotide (APAD) and Lactate (present in substrate buffer) are converted by pLDH to APADH and Pyruvate. APADH reduces the chromogenic substrate Nitro Blue Tetrazolium (NBT) using the enzyme diaphorase. This results in the formation of Nitro Blue Formazan (NBF), a deep purple soluble stain that can be measured at a wavelength of 650 nm.

Schematic representation:



Materials

- Positive control: BPRC 98 rabbit standard at 6 mg/ml total Ig.
- Negative control: Negative rabbit, rhesus or human IgG fraction, purified similarly as the samples.
- 50 ml tubes: Greiner Bio-one #22761
- 96-well flat-bottom culture plates with individual lids: Greiner Bio-one #655180
- Aluminiumfoil
- Centrifuge for culturing and spinning down RBC's: Beckman Coulter Allegra X-22R
- Centrifuge for harvesting of GIA-plates: Beckman Spinchron R Centrifuge
- Eppendorf centrifuge: Eppendorf centrifuge 5424

- Eppendorf tubes: Eppendorf Safe-lock 1.5 ml #0030 120.086
- Filters: 0.45 μm: Whatman FP30/0,45 CA-S #10462100
- Flatbed shaker: Edmund Bühler TiMix CONTROL
- Humidified box: plastic box with wet tissues or water at the bottom
- Humidified incubator: Sanyo O2/CO2 incubator
- Microscope: Zeiss Axioskop
- Platereader: Bio-Rad Model 680 Microplate Reader
- Slides: Menzel-Gläzer 76x24 mm
- 100% Methanol: Merck #1.06009.2500; CH3OH, M=32.04
- 2xCmed = 2x Complete Culture Medium:
  RPMI1640 + 20% Human serum + 30 μg/ml Gentamycin
  To prepare 200 ml 2xCmed: Mix 160 ml RPMI1640 + 40 ml Human serum + 120 μl Gentamycin. Store at 4 °C.
- APAD: Sigma #A5251; C22H28N6O14P2, M=662.44
  3-Acetylpyridine Adenine Dinucleotide
  To prepare 10 ml stock solution of 10 mg/ml: dissolve 100 mg of APAD in 10 ml distilled water. Make 50 μl aliquots and store at -20 °C.
- Diaphorase from Clostridium kluyveri: Sigma #D5540
  To prepare 30 ml stock solution of 50 units/ml: dissolve 1.500 units Diaphorase in 30 ml distilled water. Make 200 μl aliquots and store at -20 °C.
- EDTA: Merck #1.00944.1000; C10H16N2O8, M=292.25
  Ethylenediamine tetraacetic acid
  To prepare an 8 mM EDTA solution: Dissolve 116.9 mg in 40 ml RPMI1640, adjust to pH 7.5, fill up to 50 ml with RPMI1640 and sterilise through a 0.45 μm filter, store at 4 °C.
- Gentamycin: Gibco #15750-037; stock = 50 mg/ml
- Giemsa buffer: Merck #1.09468.0100; Na2HPO4*2H2O KH2PO4
  Buffer tablets pH 7.2
  To prepare 1 litre Giemsa buffer: dissolve 1 buffer tablet in 1 liter distilled water.
- Giemsa stain: Sigma #GS500
  To prepare fresh Giemsa stain: dilute 1 part Giemsa stain in 4 parts Giemsa buffer, filter through a 0.45 μm filter, use immediately.
- Heat-inactivated Human Serum A+: Bloedbank Leidsenhage, store at -20 °C.
- Human RBC O+ (multipe donors): Bloedbank Rotterdam, store at 4 °C.
- LDH-buffer: Sigma #L7022; C3H5NaO3, M=112.06
  Sodium L-lactate
  To prepare 500 ml of the buffer, mix 50 ml of 1M TRIS-HCl (pH 8.0) and 450 ml distilled water. Add 2.8 g Sodium L-Lactate and 1.25 ml Triton X-100. Mix on a magnetic stirrer at room temparature for at least 30 minutes. Make 50 ml aliquots and store at -20 °C.
- NBT: Sigma #N6639; C40H30N10O6*2Cl, M=817.64
  Nitro Blue Tetrazolium chloride
- PBS: Gibco #10010
  Phosphate Buffered Saline pH 7.4
- RPMI 1640: Gibco #52400

RPMI1640 + L-glutamine + 25 mM HEPES

Method

Timing of 2-cycle experiment

Timing is essential in a 2-cycle GIA, which depends on the stage the synchronised parasites are in. There are two time schedules you can follow:

					Development stage:
start	**d=0**	**9**	**hrs**	**t=0**	**ripe schiz.**
medium addition	**d=1**	**17**	**hrs**	**t=32**	**late throps**
	d=2				
harvest	**d=3**	**17**	**hrs**	**t=80**	**late trophs**

Or:

					Development stage:
start	**d=0**	**15**	**hrs**	**t=0**	**trophs**
	d=1				
medium addition	**d=2**	**17**	**hrs**	**t=50**	**schizonts**
	d=3				
harvest	**d=4**	**9**	**hrs**	**t=96**	**late trophs**

Preparation of uninfected RBC for use in GIA

1. Use the same batch of RBC used for the culturing of parasites in the last few days.
2. Make a RBC suspension of 1% hematocrit in 2xCmed.

Preparation of parasites

1. Count the parasitaemia in a smear stained with Giemsa, making sure all parasites are in the late thorps or schizont stage.
2. Transfer the parasite-culture to a 50 ml tube.
3. Spin for 5 minutes at 2000rpm.
4. Remove supernatant.
5. Dilute enough of the parasite-pellet in 2xCmed for 0.1% parasitaemia.
6. Add RBC 50% HT to get a 1% HT suspension.

Assay set-up

1. Use 96-well flat-bottom culture plates with individual lids.
2. Label all plates with the parasite-line used, date, initials and plate-number (numbers on both lid and plate).
3. Pipette 25 μl IgG's in triplicates (50 μl if you make a serial dilution of the IgG's, than also pipette 25 μl of plain RPMI in the other wells for serial dilution).
4. Pipette 25 μl positive IgG in triplicate.
5. Pipette 25 μl negative IgG in triplicate.
6. Pipette 25 μl plain RPMI for the schizont controls in triplicates.
7. Pipette 25 μl plain RPMI for the RBC-control.
8. Pipette 25 μl plain RPMI for the monitor-wells.
9. Add to all wells, except the RBC-controls, 25 μl parasite-suspension.
10. Add to the RBC-control-wells 25 μl RBC-suspension.
11. Pipette 25 μl 8 mM EDTA for the EDTA-control.
12. Put the plates in a humidified box (wet tissues or water at the bottom of the box), and place the lid on the box, but don't close it completely.
13. Put the boxes with the plates in a humidifed incubator at 37 °C, containing 5% CO_2, 5% O_2, 90% N_2.

14. Add 50 ul fresh medium to each well at the appropiate time (see time schedule)
15. Make a smear of 5-6 monitor-wells of one of the plates at appropiate times and stain with Giemsa (See protocol ***3.6*** *Giemsa staining*) to monitor parasite-stage.

Final start hematocrit: 1%
Final start parasitaemia: 0.1%

Harvest of assay plates

1. Make a smear of 5-6 monitor-wells of all plates and stain with Giemsa (See protocol ***3.6*** *Giemsa staining*) to check parasite-stage. Harvest at late throps or schizont stage.
2. Fill new 96-well flat-bottom culture plates with 200 μl/well of cold PBS (one plate for each assay plate).
3. Mix the contents of the wells in the assay plate thoroughly using a multichannel and transfer 100 μl to the PBS-plate (in the same format). Use same tips when possible (i.e. for each sample).
4. Spin all plates (both the original plates and the new plates) for 10 minutes at 1300g at 4 °C with low brake.
5. Remove 240 μl of supernatant without disturbing the pellet. Use same tips where possible.
6. Freeze the plates overnight at -20 °C (or until ready for pLDH-assay) to lyse erythrocytes.

LDH assay

NOTE: NBT is light sensitive, so avoid direct light and keep solutions and plates with substrate in the dark (cover with aluminium foil)

1. Thaw the plates and LDH-buffer and warm up to room temperature (10 ml LDH-buffer/plate).
2. Dissolve NBT in LDH-buffer at a concentration of 2mg/10ml. Mix gently and keep substrate in dark.
3. Add 50 μl APAD stock (10 mg/ml) to every 10 ml substrate.
4. Add 200 μl Diaphorase stock (50 units/ml) to every 10 ml substrate.
5. Use substrate immediately.
6. Add 100 μl substrate per well of the harvested plates. One plate every minute.
7. Cover with aluminium-foil and place on a flatbed shaker at 400 rpm at room temparature.
8. Incubate for 30 minutes.
9. Measure OD after 30 minutes at wavelength 655 nm (A655). One plate every minute.

Results

OD-values measured by the platereader should be exported to a csv-file.
% inhibitions can be calculated using the following formula:

$$\% \text{ inhibition} = 100\% \frac{(\text{A655 IgG sample} - \text{A655 RBC control})}{(\text{A655 Sz control} - \text{A655 RBC control})} \times 100\%$$