METHODS IN MOLECULAR MEDICINE™

Malaria Methods and Protocols

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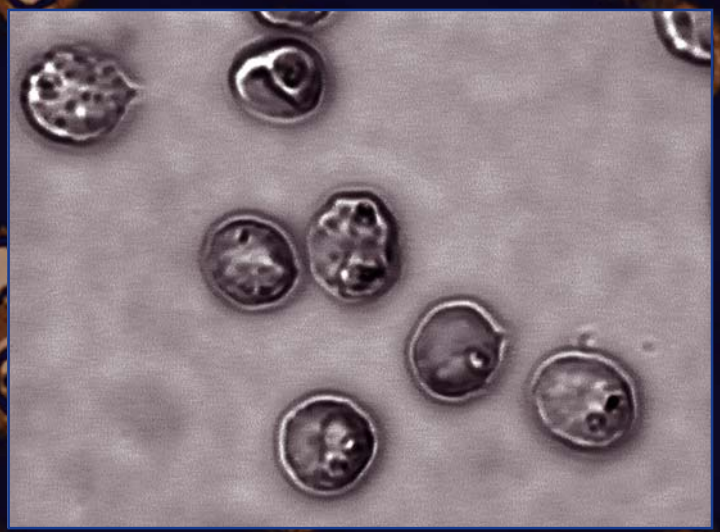

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Antibody-Dependent Cellular Inhibition Assay

Pierre Druilhe and Hasnaa Bouharoun-Tayoun

1. Introduction

The antibody-dependent cellular inhibition (ADCI) assay is designed to assess the capability of antibodies to inhibit the in vitro growth of *Plasmodium falciparum* in the presence of monocytes. Our studies have shown that antibodies that proved protective against *P. falciparum* blood stages by passive transfer in humans are unable to inhibit the parasite in vitro unless they are able to cooperate with blood monocytes ***(1,2)***. It was also shown that antibodies that were not protective in vivo had no effect on *P. falciparum* growth in the ADCI assay ***(3,4)***. The ADCI is therefore an in vitro assay the results of which reflect the protective effect of antimalarial antibodies observed under in vivo conditions in humans.

The antibodies able to cooperate with monocytes should be obviously cytophilic: IgG1 and IgG3 isotypes are efficient in ADCI while IgG2, IgG4, and IgM are not efficient. This is consistent with our findings that in sera from protected individuals, cytophilic anti-*P. falciparum* antibodies are predominant, although in nonprotected patients the antibodies produced against the parasite are mostly noncytophilic ***(5)***.

Our results suggest that ADCI likely involves the following succession of events: at the time of schizonts rupture, the contact between some merozoite surface component and cytophilic antibodies bound to monocytes via their Fc fragment triggers the release of soluble mediators which diffuse in the culture medium and block the division of surrounding intraerythrocytic parasites ***(6)***.

The major steps involved in the ADCI protocol are as follows:

1. Serum IgG preparation using ion exchange chromatography.
2. Monocyte isolation from a healthy blood donor.
3. Preparation of *P. falciparum* parasites including synchronization and schizont enrichment.
4. Parasite culture, for 96 h, in the presence of antibodies and monocytes.
5. Inhibition effect assessed by microscopic observation and parasite counting.

2. Materials

2.1. IgG Preparation

1. Tris buffer: 0.025 *M* Tris-HCl, 0.035 *M* NaCl, pH 8.8.
2. Phosphate-buffered saline (PBS), pH 7.4.
3. GF-05-Trisacryl filtration column (IBF, Biothecnics, Villeneuve La Garenne, France).

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4. DEAE-Trisacryl ion exchange chromatography column (IBF).
5. G25 Filtration column.
6. Amicon filters and tubes for protein concentration (mol wt cutoff: 50,000).
7. Sterile Millex filters, 0.22-µm pore size (Millipore Continental Water Systems, Bedford, MA).
8. Spectrophotometer equipped with ultraviolet lamp.

2.2. Monocyte Preparation

1. Heparinized blood collected from a healthy donor, 20–40 mL vol.
2. Ficoll-Hypaque density gradient (Pharmacia LKB, Uppsala, Sweden).
3. Hanks' solution supplemented with $NaHCO_3$, pH 7.0.
4. RPMI 1640 culture medium supplemented with 35 m*M* HEPES and 23 m*M* $NaHCO_3$; prepare with mineral water; store at 4°C.
5. Reagents for nonspecific esterase (NSE) staining *(7)*: fixing solution, nitrite, dye, buffer, and substrate
6. 96-Well sterile plastic plates (TPP, Switzerland).
7. Refrigerated centrifuge.
8. CO_2 incubator.
9. Inverted microscope.

2.3. Parasite Preparation

1. RPMI 1640 culture medium (*see* **Subheading 2.2., item 4**).
2. 10% Albumax stock solution; store at 4°C for up to 1 mo.
3. 5% Sorbitol for parasite synchronization.
4. Plasmagel for schizont enrichment.
5. Reagents for fixing and staining of thin smears: methanol, eosine, methylene blue.

3. Methods

3.1. IgG Preparation

IgGs are extracted from human sera (*see* **Note 1**) as follows:

1. Dilute the serum at a ratio of 1 to 3 in Tris-HCl buffer.
2. Filter the diluted serum through a GF-05 Trisacryl gel filtration column previously equilibrated in the Tris-HCl buffer. Ensure that the ratio of serum to filtration gel is 1 vol of undiluted serum to 4 vol of GF-05 gel.
3. Pool the protein-containing fractions
4. Load over a diethylaminoethanol (DEAE)-Trisacryl ion exchange chromatography column previously equilibrated with Tris buffer. Ensure that the ratio of serum to filtration gel is 1 vol of undiluted serum to 4 vol of DEAE gel.
5. Collect fractions of 1 mL volume.
6. Measure the optical density (OD) of each fraction using a 280-nm filter.
7. Calculate the IgG concentration as follows:

$$\text{IgG concentration (mg/mL)} = \frac{\text{OD 280 nm}}{1.4}$$

8. Pool the fractions containing IgGs.
9. Concentrate the IgG solution using Amicon filters. Amicon filters are first soaked in distilled water for 1 h and than adapted to special tubes in which the IgG solution is added.
10. Centrifuge the tubes at 876*g* for 2 h at 4°C. This usually leads to a 25-fold concentration.
11. Perform a final step of gel filtration using a G25 column previously equilibrated in RPMI culture medium.

12. Collect the IgG fractions in RPMI.
13. Measure the optical density (OD) of each fraction using a 280-nm filter.
14. Calculate the IgG concentration.
15. Pool the fractions containing IgGs.
16. Sterilize the IgG fractions by filtration through 0.22-µm pore size filters.
17. Store the sterile IgG solution at 4°C for up to 1 mo (or add Albumax for longer storage—but this is not recommended).

3.2. Monocyte Preparation

The procedure for monocyte preparation is based on that described by Boyum ***(8)*** and includes the following steps:

1. Dilute the heparinized blood threefold in Hanks' solution.
2. Carefully layer 2 vol of diluted blood onto 1 vol of Ficoll-Hypaque (maximum volume of 20 mL of diluted blood per tube).
3. Centrifuge at 560*g* for 20 min at 20°C.
4. Remove the mononuclear cell layer at the Ficoll-Hypaque/plasma interface.
5. Add 45 mL of Hanks' solution to the mononuclear cell suspension.
6. Centrifuge at 1000*g* for 15 min at 20°C.
7. Carefully resuspend the pelleted cells in 45 mL of Hanks' solution.
8. Centrifuge again at 1000*g* for 15 min at 20°C. Repeat this washing step twice more.
9. Finally, centrifuge at 180*g* for 6 min at 20ºC, to remove any platelets that remain in the supernatant.
10. Resuspend the mononoclear cells in 2 mL of RPMI.
11. Calculate the mononuclear cell concentration (i.e., lymphocytes plus monocytes) in the cell suspension: dilute a 20-µL aliquot of the cell suspension threefold in RPMI and count cell numbers using a hemocytometer (e.g., Malassez type).
12. Determine the number of monocytes using the nonspecific esterase (NSE) staining technique:
    a. In microtube A, add 40 µL of mononuclear cell suspension to 40 µL of fixing solution.
    b. In microtube B, mix the NSE staining reagents in the following order: 60 µL of nitrite, 60 µL of dye,180 µL of buffer, and 30 µL of substrate
    c. Add the mixture in microtube B to the cells in microtube A.
    d. Take a 20-µL sample of the stained cells and measure the proportion of monocytes: lymphocytes: monocytes will be colored in brown, whereas the lymphocytes will be uncolored. Usually the proportion of monocytes is 10–20% of the total mononuclear cells.
13. Adjust the cell suspension to a concentration of 2×10^5 monocytes per 100 µL, with RPMI.
14. Aliquot the cell suspension in a 96-well plate at 100 µL per well.
15. Incubate for 90 min at 37°C, 5% CO_2. During this incubation, monocytes will adhere to the plastic.
16. Remove the nonadherent cells and wash the monocytes by adding, and thoroughly removing, 200 µL of RPMI in each well.
17. Repeat this washing procedure three times in order to remove all the nonadherent cells.
18. At least 95% of the recovered cells will be monocytes. Control for the cell appearance and the relative homogeneity of cell distribution in the different wells by observation using an inverted microscope (*see* **Notes 2–4**).

3.3. Parasite Preparation

P. falciparum strains are cultivated in RPMI 1640 supplemented with 0.5% Albumax.

Parasites are synchronized by Sorbitol treatments ***(9)*** as follows:

1. Dilute the sorbitol stock to 5% in mineral water.
2. Centrifuge the asynchronous parasite culture suspension at 250*g* for 10 min at 20°C.
3. Resuspend the pellet in the 5% sorbitol solution. This will lead to the selective lysis of schizont infected RBC without any effect on the rings and young trophozoites.

When required, schizonts are enriched by flotation on plasmagel ***(10)*** as follows:

1. Centrifuge cultures containing asynchronous parasites at 250*g* for 10 min at 20°C
2. Resuspend the pellet at a final concentration of 20% red blood cells (RBC), 30% RPMI, 50% plasmagel.
3. Incubate at 37°C for 30 min. Schizont-infected RBC will remain in the supernatant, whereas young trophozoite-infected and -uninfected RBC will sediment.
4. Collect carefully the supernatant, by centrifugation at 250*g* for 10 min at 20°C.
5. Prepare a thin smear from the pelleted cells, stain, and determine the parasitemia by microscopic examination.
6. Usually, using this method, synchronous schizont-infected RBC are recovered at ~70% parasitemia.

For the ADCI assay, synchronized early schizont parasites are used. Usually the parasitemia is 0.5–1.0% and the hematocrit 4%.

3.4. The ADCI Assay

1. After the last washing step, add in each monocyte containing well:
   a. 40 µL of RPMI supplemented with 0.5% Albumax (culture medium).
   b. 10 µL of the antibody solution to be tested. Usually the IgGs are used at 10% of their original concentration in the serum (~20 mg/mL for adults from hyperendemic areas, and ~12 mg/mL for children from endemic area and primary attack patients) (*see* **Note 5**).
   c. 50 µL of parasite culture, at 0.5% parasitemia and 4% hematocrit.
2. Control wells consist of the following:
   a. Monocytes (MN) and parasites with normal IgG (N IgG) prepared from the serum of a donor with no history of malaria.
   b. Parasite culture with IgG to be tested without MN.
3. Maintain the culture at 37°C for 96 h in a candle-jar (or a low O_2, 5% CO_2 incubator).
4. Add 50 µL of culture medium to each well after 48 and 72 h.
5. Remove the supernatant after 96 h. Prepare thin smears from each well, stain, and determine the parasitemia by microscopic examination. In order to ensure a relative precision in the parasite counting, a minimum of 50,000 RBC should be counted and the percentage of infected RBC calculated (*see* **Notes 6** and **7**).
6. Calculate the specific growth inhibitory index (SGI), taking into account the possible inhibition induced by monocytes or antibodies alone:

$$\text{SGI} = 100 \times 1 - \frac{\text{percent parasitemia with MN and Abs/percent parasitemia with Abs}}{\text{percent parasitemia with MN + N IgG/percent parasitemia with N IgG}}$$

4. Notes

1. IgG preparation from sera to be tested is an essential step because we have frequently observed a nonantibody-dependent inhibition of parasite growth when unfractionated sera were used, probably due to oxidized lipids.
2. Monocyte (MN) function in ADCI is dependent upon several factors such as water used to prepare RPMI 1640. Highly purified water, such as Millipore water, although adequate for parasite culturing, leads to a poor yield in the number of MN recovered after adherence

to the plastic wells. On the other hand, water which contains traces of minerals, such as commercially available Volvic water, or glass-distilled water, provide consistently a good monocyte function.

3. Improved monocyte adherence can be obtained by coating the culture wells with fibronectin, that is, coating with autologous plasma from the MN donor, followed by washing with RPMI 1640, prior to incubation with mononuclear cells.
4. MN from subjects with a viral infection (e.g., influenza) are frequently able to induce a non-IgG-dependent inhibition of parasite growth. This nonspecific inhibition effect could prevent the observation of the IgG-dependent inhibition in ADCI. Therefore, MN donors suspected of having a viral infection, or who have had fever in the past 8 d, should be avoided. The results from ADCI are not reliable when the direct effect of MN alone is greater than 50% inhibition. The preparation of MN in medium containing heterologous serum, such as FCS, results in the differentiation of MN, their progressive transformation into macrophages which have lost their ADCI-promoting effect.
5. If required, murine IgG can be tested in ADCI with human MN. The IgG2a isotype is able to bind to the human Fc γ receptor II present on monocytes shown to be involved in the ADCI mechanism ***(6)***.
6. A possible variation of the ADCI assay is the assessment of a competition effect between protective cytophilic antibodies (adults from hyperendemic area) directed to the merozoite surface antigens, and nonprotective antibodies (children from endemic area and primary attack patients) which recognize the same antigens but are not able to trigger the monocyte activation because they do not bind to Fc gamma receptors. Therefore noncytophilc Ig directed to the "critical" antigens may block the ADCI effect of protective antibodies. Each IgG fraction should be used at 10% of its original concentration in the serum.
7. The ADCI assay protocol can be modified and performed as a two-step ADCI with short-term activation of monocytes according to the following procedure:
   a. Incubate MN for 12–18 h with test Ig and synchronous mature schizonts infected RBC, at 5–10% parasitemia. During this first culture time, infected RBC rupture occurs and merozoites are released.
   b. Collect supernatants from each well and centrifuge them at 7000*g*.
   c. Distribute the supernatants in a 96-well plate, at 100 µL per well.
   d. Add to each well 100 µL of *P. falciparum* asynchronous culture containing fresh medium, at 0.5–1% parasitemia, 5% hematocrit (particular care is taken to reduce to a minimum the leukocyte contamination of the RBC preparation used for this second culture).
   e. At 36 h of culture, add 1 mCi of [^{3}H]hypoxanthine to each well.
   f. At 48 h of culture, harvest cells and estimate [^{3}H] uptake by counting in a liquid scintillation counter.

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