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Use of magnetically purified *Plasmodium falciparum* parasites improves the accuracy of erythrocyte invasion assays

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Abstract

Merozoite invasion of erythrocytes is a crucial step for the asexual cycle of *Plasmodium falciparum*. Multiple invasion pathways, which involve different ligand-receptor interactions, have been identified in *P. falciparum* by examining the entry of purified parasite into erythrocytes with different surface receptors, either mutant or under different enzyme treatments. The most critical step for a successful invasion assay is the isolation of erythrocytes infected with viable schizonts. Here, we applied a magnetic column to purify the schizonts for the erythrocyte invasion assay. Comparing to Percoll-sorbitol purification method, this modified approach showed great improvement on reproducibility and reliability of invasion assay, particularly for short-term, culture-adapted parasite isolates. The magnetic purification method is an excellent alternative for parasite isolates that do not tolerate or with unknown sensitivity to Percoll-sorbitol exposure.

Keywords

Plasmodium falciparum; parasite purification; erythrocyte invasion; magnetic column

1. Introduction

The invasion of erythrocytes by *Plasmodium falciparum* merozoites is a complex process involving multiple pathways, which rely on a number of specific interactions between parasite ligands and erythrocyte receptors (Gaur, et al., 2004). Blocking erythrocyte invasion interrupts the parasite life cycle and is therefore a major aim of vaccine development programs. Although some parasite ligands and erythrocyte receptors have been characterized, the molecular basis of the invasion process is still not fully understood (Gaur, et al., 2004). To study *P. falciparum* invasion of erythrocytes, a reliable method to measure invasion efficiency is essential. The various invasion assay protocols currently in use can be characterized by three general steps: (i) purification of trophozoites and schizonts, (ii) addition of these purified parasites to an erythrocyte suspension of known density, and (iii) quantification of parasitemia after allowing sufficient time -approximately 20 hours- for parasite invasion and growth in target erythrocytes (Gaur, et al., 2003). The most critical step for a successful invasion assay is the isolation of erythrocytes infected with viable trophozoites and schizonts ('mature parasitized erythrocytes').

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Traditional parasite synchronization methods rely on the differential sugar and anion permeability of erythrocytes infected with different stages of parasites. Specifically, the uptake of D-sorbitol by mature parasitized erythrocytes leads to their osmotic lysis, which produces a relatively synchronous culture of immature ring-stage parasites (Lambros and Vanderberg, 1979). Mature parasitized erythrocytes can then be obtained by cultivating the synchronized ring-stage parasites for approximately 24 hours. Although useful for parasite synchronization, the sorbitol lysis method does not remove uninfected erythrocytes and therefore cannot produce pure parasite preparations. While gelatin flotation is a widely used method to enrich for mature parasitized erythrocytes, this method also does not produce pure parasite preparations (Goodyer, et al., 1994). Instead, Percoll-sorbitol density gradient centrifugation is widely used to purify mature parasitized erythrocytes for invasion assays (Ginsburg, et al., 1987, Rivadeneira, et al., 1983). The propensity of mature parasitized erythrocytes to take up sorbitol and anions in combination with their lower density relative to ring-stage parasitized erythrocytes and uninfected erythrocytes enables clear separation of these populations following centrifugation (Rivadeneira, et al., 1983). Though efficient and capable of yielding nearly 100% pure parasite populations, most Percoll-based protocols require high-speed centrifugation and multiple washing steps to prevent lysis upon re-introduction of the parasites to culture medium. Despite the successful application of this purification method in many invasion studies, some long-term, culture-adapted *P. falciparum* parasite lines such as W2mef (Reed, et al., 2000) cannot be efficiently purified by this method and many short-term, culture-adapted parasite isolates purified by this method resulted in low erythrocyte invasion efficiencies (see later).

To more accurately measure the invasion efficiency of those parasites whose viability appeared to be reduced by the Percoll-sorbitol method, we used a magnetic column to purify mature parasitized erythrocytes. We then compared the invasion efficiency of parasites purified by magnetic column to those purified by the Percoll-sorbitol method. The magnetic column method exploits the presence of haemozoin (produced from the breakdown of Fe(II)-containing haemoglobin) in mature parasitized erythrocytes. When a parasitized erythrocyte culture is applied to the column in the presence of a magnetic field, mature parasitized erythrocytes are retained, while ring stage-infected and uninfected erythrocytes pass through the column. Upon removal of the magnetic field, mature parasitized erythrocytes can then be eluted as a highly pure parasite population. Previous studies using MACS® 25 LD magnetic columns (Miltenyi Biotec, Auburn, CA, USA) have successfully separated various mature stages of *Plasmodium* species from patient blood samples and *in vitro* cultures (Ribaut, et al., 2008, Trang, et al., 2004).

2. Materials and methods

In this study, we used both long-term, culture-adapted *P. falciparum* lines (Dd2, MTS1, M5, and 418) (Mu, et al., 2003) and short-term, culture-adapted *P. falciparum* isolates obtained from Cambodian patients with malaria. Blood collections from patients were approved by the NIAID Institutional Review Board and the Cambodian National Ethics Committee for Health Research. Parasite cultures were maintained in human O⁺ erythrocytes at 2% hematocrit as described (Raj, et al., 2009). Invasion assays were carried out through a modification of previously described methods (Ribaut, et al., 2008, Trang, et al., 2004). Briefly, 50 mL of a *P. falciparum* culture suspension (3%-8% parasitemia, 2% hematocrit) was split into two volumes. One volume was used in the Percoll-sorbitol purification method and the other in the MACS® purification method. Percoll-sorbitol purification was performed using a 70%:40% Percoll gradient as described (Gaur, et al., 2003). For MACS® purification, parasitized culture suspensions were adjusted to 1% hematocrit and then applied to MACS® 25LD magnetic columns as described previously (Ribaut, et al., 2008). After elution, the concentration of pure parasitized erythrocytes was estimated using a hematocytometer. Approximately 6×10^5

parasitized erythrocytes and 4×10^7 uninfected target erythrocytes were combined in 200 μ L of culture medium and added to a single flat-bottom, 96-well microtiter plate (Corning Costar, Lowell, MA). Assay plates were incubated at 37°C for 40 hours in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Neuraminidase, trypsin, and chymotrypsin treatments of target erythrocytes were carried out as described previously (Gaur, et al., 2003). Invasion assays were performed in duplicate on three separate occasions. Following incubation, mature parasitized erythrocyte culture suspensions were washed twice with PBS, stained with 2 μ g/mL of ethidium bromide for 30 minutes, and washed again. Parasitemia was then determined by flow cytometry using a Becton-Dickinson FACS Calibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA) as described previously (Fairhurst, et al., 2005).

3. Results and discussion

We first compared the invasion rates of four long-term, culture-adapted *P. falciparum* strains (Dd2, MT/S1, M5, and 418 (Mu, et al., 2003)) that were purified by either the magnetic column or Percoll-sorbitol method (Figure 1). In order to minimize variability, parasite purifications by the two methods were performed in parallel. Rhesus monkey erythrocytes, which are completely resistant to invasion by *P. falciparum*, were included as a negative control to correct for invasion into residual uninfected erythrocytes following the purification steps. For each parasite line, we found that parasites purified by magnetic column had a similar invasion rate to those purified by Percoll-sorbitol (paired T-test, $P > 0.1$) (Figure 1). These results indicate that magnetic column method is a reliable and effective alternative to the Percoll-sorbitol method for purification of long-term, culture-adapted *P. falciparum* lines for use in invasion assays.

We then investigated the invasion rates of five short-term (about 4-5 weeks *in vitro* culture), culture-adapted parasites from Cambodia (CP191, CP195, CP 271, CP286, CP315) that seemed to invade poorly after being purified by the Percoll-sorbitol method. Although sufficient numbers of mature parasitized erythrocytes could be obtained by the Percoll-sorbitol method, the ring-stage parasitemias of enzyme-treated and untreated erythrocytes were very low (Figure 2), making it difficult to accurately estimate the true invasion rates of these parasite isolates. In contrast, the magnetic column method consistently produced higher invasion rates than the Percoll-sorbitol method (paired T-test, $P < 0.05$) (Figure 2). These parasite isolates were adapted to culture for only 4-5 weeks after obtaining them from patients, and may have been more sensitive to Percoll-sorbitol treatment. Compared to newly adapted parasites, long-term adapted parasites seems to invade erythrocytes more efficiently and to be less sensitive to sorbitol. This is likely due to some unknown compensatory changes that occurred during the *in vitro* culture adaptation process, which presents an interesting issue that requires further investigation.

These results indicate that the magnetic column method is superior to the Percoll-sorbitol method for purifying viable parasite isolates for use in invasion studies, at least for some parasite isolates.

In summary, although the Percoll-sorbitol method can produce viable *P. falciparum* schizonts for the majority of long-term, culture-adapted parasites, this method did not seem to work effectively for 70% of the short-term, culture-adapted Cambodian parasite isolates that we tested (data not shown). In invasion assays, the magnetic column method produced more accurate results than the Percoll-sorbitol method. We propose that the magnetic column method should be used to prepare parasites for erythrocyte invasion assays if the sensitivity of a parasite isolate to Percoll-sorbitol treatment is unknown.

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Figure 1. Erythrocyte invasion rates of four long-term, culture adapted *P. falciparum* lines

Four different *P. falciparum* strains (Dd2, MTS1, M5, and 418) adapted to long-term culture were simultaneously purified by either the Percoll-sorbitol (Percoll) or the magnetic column (MACS) method. Purified parasitized erythrocytes were added to untreated or enzyme-treated target erythrocytes and then cultured to allow time for merozoites to invade erythrocytes and develop to mature stages. Invasion rates were obtained by normalizing the parasitemias of enzyme-treated erythrocytes to those of untreated erythrocytes.

Figure 2. Erythrocyte invasion rates of five short-term, culture-adapted *P. falciparum* isolates

Five parasite isolates (CP191, CP195, CP271, CP286, CP315) were obtained from Cambodian patients with malaria. After 4-5 weeks of *in vitro* culture, parasitized erythrocytes were purified by either the Percoll-sorbitol (Percoll) or magnetic column (MACS) method. Invasion assays were performed as outlined in the legend to Figure 1, and the parasitemias were measured and compared.