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Antibodies to *Plasmodium* circumsporozoite protein (CSP) inhibit sporozoite's cell traversal activity

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Abstract

Plasmodium sporozoites are deposited in the skin of the mammalian host by *Anopheles* mosquitoes. To continue the life cycle, the sporozoites have to invade the host's hepatocytes, where they transform into exoerythrocytic forms (EEFs) inside a parasitophorous vacuole. During their route from the skin to the liver, the parasites traverse the capillary epithelium in the dermis to enter the blood circulation, and cross the endothelium of liver sinusoids to enter the parenchyma. Cell traversal by sporozoites is usually measured by quantifying dyes that enter or are released from cells during incubation with salivary gland sporozoites. These methods do not distinguish cell traversal from cell wounding. Here we validate an assay that quantifies cell traversal of sporozoites through monolayers of MDCK cells that form tight junctions. We compared cell traversal of wt sporozoites and of parasites lacking the Type I membrane protein TLP (TRAP-like protein) previously implicated in cell traversal. We provide direct evidence that TLP ko sporozoites are defective in cell traversal and that they are retained inside the MDCK cytoplasm. We then used the MDCK assay to study the effect of a monoclonal antibody (3D11) to the circumsporozoite protein (CSP) on the parasite's cell traversal. We show that 3D11 inhibits cell traversal at nanomolar concentrations. We conclude that antibodies elicited by CSP-based vaccines are likely to inhibit the migration of sporozoites from the skin to the liver.

Keywords

Plasmodium; sporozoites; MDCK cell assay; TRAP-like protein (TLP); cell traversal

1. Introduction

The sporozoites of malaria parasites undergo profound genetic changes while they move from the mosquito oocysts into the salivary glands (Matuschewski, 2006), where they can remain dormant for days (Zhang et al., 2010). After delivery into the skin of the mammalian host by mosquito bite, the parasites start a long journey to the liver, where they enter hepatocytes and develop into the exoerythrocytic forms. During this migration the parasites need to cross the vascular endothelium to enter the blood or lymphatic circulation, and the

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endothelium of liver sinusoids to enter the parenchyma (Mota et al., 2001; Amino et al., 2008; Ejigiri and Sinnis, 2009). Sporozoite migration and infection of hepatocytes are achieved by the concerted activity of an actin-myosin motor located beneath the parasite's plasma membrane (Kappe et al., 2004; Dowse and Soldati, 2004) and by products of specialized secretory organelles named rhoptries, dense granules and micronemes (Carruthers and Sibley, 1997). Sporozoites do not have flagella, cilia or lamellipodia, nevertheless they use an actin/myosin motor to move very rapidly over solid substrates by gliding motility (Heintzelman, 2006; Tardieux and Ménard, 2008; Daher and Soldati-Favre, 2009; Munter et al., 2009). The first sporozoite membrane protein shown to link the motor to the extracellular substrate was the thrombospondin related anonymous protein (TRAP) [Sultan et al., 1997]. In the absence of TRAP sporozoites do not glide. Other members of the TRAP family were identified later (Lacroix and Menard, 2008), including TLP (that is the subject of this paper; Moreira et al., 2008; Heiss et al., 2008). All members bear a cytoplasmic tail that is acidic and interchangeable. This tail contains a sub-terminal tryptophan that is essential for motility (Kappe et al., 1999). The extracellular domains of TRAP family members consist of two adhesive modules that bind to substrates: the thrombospondin the type I repeat and the A domain.

Two methods are generally used to study cell traversal. In the calcein green assay, target cells loaded with a fluorescent dye are incubated with sporozoites and the amounts of fluorescence released into the supernatant are measured (Coppi et al., 2007). This assay was used in the studies of the TLP knockout (Moreira et al., 2008). However, the calcein assay measures cell wounding rather than cell traversal. A second cell traversal assay uses polarized monolayers of MDCK cells seeded between two chambers (Mota et al., 2001). The sporozoites are placed in the upper chamber, and after an appropriate time the parasites in the bottom chamber are counted. As described, this assay does not exclude the possibility that sporozoites migrate between cell junctions as is the case of *Toxoplasma* tachyzoites (Barragan et al., 2005). Here we ruled this out and used the MDCK assay to confirm the role of TLP in cell traversal. In addition, we studied the effect of antibodies to the sporozoite's circumsporozoite protein (CSP) on their ability to traverse cells.

2. Material and methods

2.1. Parasites, cells and antibodies

The *Plasmodium berghei* wt and the mutant lacking TLP [*PbTLP ko*] were described elsewhere (Moreira et al., 2008). The MDCK and HepG2 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and 1 mM glutamine (DMEM/FCS). The following antibodies were used: monoclonal antibody (mAb), directed against the repeat region of *P. berghei* CSP (Yoshida et al., 1980) and the anti-occludin monoclonal antibody (Invitrogen).

2.2. Mosquito infection with *PbTLP ko* and wt parasites

All procedures involving animals were performed according to US National Institutes of Health guidelines, as approved by the Animal Care and Use Committee of the New York University School of Medicine laboratory animal protocol #010202-01. Wild type (wt) and *PbTLP ko* parasite cycles were performed as described (Moreira et al., 2008). Briefly, *Anopheles stephensi* mosquitoes were reared at 27°C and 80% humidity under a 12/12 h light/dark cycle, and adults were fed on 10% sucrose solution. The *A. stephensi* mosquitoes were fed on anaesthetized Swiss Webster mice infected with wt parasites or with the *PbTLP ko*. The mosquitoes were rinsed in 70% ethanol, washed in medium, and the salivary glands removed. The glands were gently ground in a tissue homogenizer, centrifuged at 800 rpm

for 4 min to remove mosquito debris, and sporozoites were counted in a haemocytometer and kept on ice in complete medium (DMEM containing 10% FCS) until use.

2.3. Cell traversal assay in vitro

The ability of mutant and wt sporozoites to cross cell layers was determined by a modification of the method described by Mota et al. (2001). MDCK cells (10^5) were cultured in 3- μ m pore diameter Transwell filters (Costar) for 3 days to form a continuous monolayer. The wells underneath the filters either were left empty or 2×10^5 HepG2 cells were placed in the lower chamber. Sporozoites (10^5) were then added to the filter inset. After 1h the filter was removed and the sporozoites that crossed the MDCK monolayer were collected from the bottom well and counted. The wells that contained HepG2 cells were incubated for additional 48 h, and the HepG2 cells removed from wells. The parasite's RNA was then quantified by real-time PCR (Bruña-Romero et al., 2001). Other MDCK-containing filters were fixed with paraformaldehyde, embedded in paraffin, sectioned and stained with 3D11, and intra-cellular parasites counted. In addition we counted directly sporozoites inside and outside of the monolayer by another approach, i.e., paraformaldehyde-fixing followed by permeabilization of the MDCK cells (Renia et al., 1988). The monolayers were stained for the tight junction marker occludin following manufacturer's instructions. Trans-monolayer Electrical Resistance (TER) was measured across the monolayer during the transmigration of sporozoites using an ohmmeter (World Precision Instruments, Bradenton, FL).

2.4. Gliding motility assay

Gliding motility assays were performed as described (Coppi et al., 2006). Briefly, sporozoites were added to Lab-Tek wells pre-coated with 3D11 for 1h at 37°C. After removal of the medium, fixative was added, followed by the biotinylated 3D11 and streptavidin-FITC (Zymed) to visualize the CSP-containing trails. Gliding motility was quantified by counting the number of sporozoites associated with trails and the number of circles in each trail.

2.5. Preparation of 3D11 Fab

Monovalent Fab was prepared using the Fab micro preparation kit (Pierce), following manufacturer's instructions. Briefly, immobilized papain was equilibrated with Fab digestion buffer. The IgG sample was desalted using Zeba desalt spin column. Desalted IgG was incubated with immobilized papain and digestion reaction was performed for 5–6 h with an end-over-end mixer at 37°C. Fc fragments and remaining intact antibodies were removed by passing the sample through Agarose-protein-G column.

2.6. Treatment of *P. berghei* sporozoites with 3D11

P. berghei sporozoites, 5×10^4 for gliding motility experiments or 10^5 for the cell traversal assay were pre-incubated for 30 min at room temperature in medium containing 10% FCS and variable concentrations of 3D11. As controls, sporozoites were incubated in medium in the absence of antibodies, or added to wells in the presence of 1 μ M cytochalasin D to inhibit gliding motility.

2.7. Statistical Analysis

Results are shown as means $\pm$ SD or percentage $\pm$ SD. Unpaired two-tailed Student's *t*-test was used to determine the statistical significance.

3. Results

3.1. PbTLP ko sporozoites are defective in cell traversal

As shown in Fig. 1A and 1B about 70% fewer *PbTLP* ko sporozoites than wt crossed the MDCK monolayer. As controls, we verified that MDCK cells formed tight junction visually (Fig. 1C) and were polarized by measuring the Transmonolayer Electrical Resistance (TER). The TER did not change during the migration of the sporozoites (Fig. 1D), as would be expected if the parasites disrupted the junctions. To determine whether the mutant parasites were retained inside the cytoplasm of MDCK cells, we sectioned the monolayer, stained the sections with 3D11, and counted the parasites inside the cytoplasm of the MDCK cells. We found that 70% more *PbTLP* ko than wt sporozoites were inside the cytoplasm of the MDCK cells (Fig. 2A). Representative microscopy sections are shown in Fig. 2C and 2D. This result was confirmed by counting sporozoites inside and outside of the MDCK cells as described by Renia et al. (1988). Again, 69% more mutants than wt were inside the MDCK cells (Fig. 2B). As an additional control for the integrity of the monolayer, we added large numbers of motile *Trypanosoma brucei* to the upper chamber, and found none in the bottom chamber (data not shown). These results provide direct evidence that TLP plays an important role in *Plasmodium* sporozoite passage through cell barriers. It also validate the MDCK assay as a reliable method to measure cell traversal.

3.2. Comparison of the capacity of 3D11 IgG to inhibit cell traversal and gliding

P. berghei sporozoites were incubated with different concentrations of 3D11 IgG for 30 min at room temperature and used for cell traversal or gliding motility assays. 3D11 significantly inhibited the cell traversal ($p = 0.003$). Gliding motility was significantly inhibited ($p = 0.02$) only when comparing the numbers of >10 circles generated by antibody- treated and non- treated sporozoites. Both gliding and cell traversal were abolished at 50 $\mu\text{g/ml}$ (Fig. 3A and B). However, even at 50 $\mu\text{g/ml}$ the monovalent Fab fragments of 3D11 did not inhibit significantly cell crossing (data not shown).

4. Discussion

Here we validate the MDCK assay to measure cell traversal by sporozoites and use it to support the findings of Moreira et al., (2008) suggesting that TLP plays a role in cell traversal. To this end, we compared the ability of wt and TLP ko sporozoites to cross the monolayer of MDCK cells that separates two chambers. We found that significantly fewer TLP ko traverse the monolayer. To ensure that the MDCK cells formed tight junctions, we measured the TER of the MDCKs during sporozoites migration. The TER did not change. We then used two methods to demonstrate that TLP ko parasites were retained inside the cytoplasm of the MDCK cells: First, we sectioned the monolayer, stained the parasites with antibodies to CSP, and counted those inside the MDCK cells; second, we directly counted the inside/outside parasites as described in Renia et al. (1988). By either method we documented the greater retention of the TLP kos in the cytoplasm of the MDCK cells as compared to wt. We conclude that TLP indeed plays an important role in cell traversal. Some TLP ko sporozoites, however, crossed the monolayer. We speculate that an additional sporozoite surface molecule, perhaps TRAP itself, complements the TLP function. One key question, however, remains to be elucidated. Since gliding motility is strictly substrate-dependent, what is the nature of the substrate(s) used by sporozoites for moving rapidly through the cell cytoplasm? Cytoskeletal elements such as F-actin and tubulin are obvious candidates to interact with adhesive domains of TLP (and TRAP?), but this remains to be investigated.

The monoclonal antibody 3D11 abolished cell traversal at a concentration of 0.3 μ M and significant inhibition was detected at 100 times lower concentrations. As previously reported (Stewart et al., 1986) gliding motility was also inhibited by 3D11 although its effect was less striking (Fig. 3). One possible reason is that gliding motility is very difficult to measure with precision. The trajectories of sporozoites on glass or plastic slides vary widely. While most exhibit a stop-go circular gliding, some have straight unpredictable directions, others move in a pendulum fashion, and some remain attached and make waving movements. On Matrigel, the movement is circular but three-dimensionally it is helical (Amino et al., 2008). To bypass these complicated problems, gliding motility is usually measured indirectly by enumerating CSP trails left on the glass slides during movement. Therefore what is reported as “gliding motility” is in reality an imperfect measurement of the parasite’s secretion of CSP during gliding. Most important, gliding on glass or on artificial matrices is unlikely to reflect sporozoite’s movement *in vivo* between cells or over cell surfaces.

Our findings do not explain why antibodies to CSP interfere with sporozoites cell traversal. The antibodies do cross-link CSP and lead to “circumsporozoite protein reaction” a thread-like precipitate that increases in length towards the posterior end of the parasite (Vanderberg et al., 1969). The monovalent Fab fragments do not trigger the CSP reaction (Potocnjak et al., 1980) and they do not prevent cell crossing at high concentrations. Perhaps CSP cross-linking inhibits the actin-myosin motor leading to the paralysis of the parasite. This is speculative because CSP has no direct connection with the parasite’s cytoplasm; it is attached to the sporozoite’s plasma membrane by a glycosylphosphatidylinositol (GPI) anchor that only crosses the outer membrane of the bilayer. Nevertheless, it has been argued that the association of GPI anchors with lipid rafts in prions and in the complement receptor CD59 is associated with signaling properties (Taylor and Hooper, 2006; Kimberley et al., 2007).

Finally, we point out that CSP is a component of a human vaccine named RTS,S that protected about 50% of individuals in a recent phase III trials in Africa (Agnandji et al., 2011). Protection is at least in part mediated by antibodies against CSP (Cohen et al., 2010) known to inhibit the attachment phase of sporozoites infection of hepatocytes (Yoshida et al., 1980; Potocnjak et al., 1980). The present findings highlight an additional effector mechanism of anti-CSP antibodies: they inhibit cell traversal and should interfere with the migration of the parasites from the host’s skin to the liver.

Highlights

- New assay for sporozoites cell traversal
- TLP is required for cell traversal of *Plasmodium* sporozoites
- Antibodies to CSP inhibit cell traversal of *Plasmodium* sporozoites

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Fig. 1. *PbTLP ko* sporozoites are impaired in cell-passage activity

Wild type and *PbTLP ko* salivary gland sporozoites were added to filter inset and incubated for 1 h at 37°C. (A) The percentage of sporozoites that cross the MDCK monolayer and are present in the bottom of the well was counted. (B) Sporozoites that cross the MDCK monolayer and develop into EEFs inside HepG2 cells seeded in the bottom chamber were quantified by real time PCR. (C) Occludin staining shows that MDCK cells formed tight junctions. (D) TER was relatively constant during sporozoites migration. Data (mean $\pm$ SD) are from quadruplicates analyzed by unpaired two-tailed Student's *t*-test.

Fig. 2. Greater number of *PbTLP* ko sporozoites are retained in the cytoplasm of MDCK cells compared to wt

(A) The number of wt and *PbTLP* ko sporozoites found in the cytoplasm of MDCK cells. (B) The number of wt and *PbTLP* ko sporozoites present in the MDCK cells estimated by inside/outside staining. (C) Photograph of wt MDCK filter section. (D) Photograph of *PbTLP* ko MDCK filter section. Data (mean $\pm$ SD) are from quadruplicates analyzed by unpaired two-tailed Student's *t*-test.

Fig. 3. Monoclonal antibody 3D11 inhibits cell traversal and gliding motility of sporozoite
 (A) 3D11 significantly inhibits cell traversal ($p = 0.003$). (B) Gliding motility was also decreased significantly when comparing the number of >10 circles of treated and nontreated sporozoites ($p = 0.02$). Total number of circles is presented on top of the bar. Data (mean $\pm$ SD) are from duplicates.