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Plasmodium yoelii: Contribution of oocysts melanization to natural refractoriness in *Anopheles dirus*

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Abstract

It is well known that *Anopheles dirus* is naturally refractory to rodent malaria parasite, *Plasmodium yoelii,* but the mechanism is still largely unknown. Here, we found that some *P. yoelii* taken into *An. dirus* could develop into oocysts, but oocysts were partially melanized at 7 days and completely melanized at 15 days post-infectious blood meal. Transmission electronic microscopy could find the melanized *P. yoelii* oocysts in *An. dirus* as early as 5 days post-infection, with a few haemocytes attaching to the melanized oocysts, indicating a typical humoral melanization reaction. Although the change of protein pattern at 24 h post-infection suggested that other unknown mechanisms and/or factors might be involved in killing ookinetes, our data implied that oocysts melanization was one of the mechanisms of *An. dirus* to block *P. yoelii* development. In addition, activity of phenoloxidase, such as monophenol oxidase and *o*-diphenoloxidase, in haemolymph of *An. dirus* fed on infectious blood meal was much higher than that of mosquitoes fed on 5% glucose or normal mouse blood ($p < 0.05$), implying the possible role of PO in oocysts melanization by *An. dirus*.

Index Descriptors and Abbreviations: *Plasmodium yoelii*; Protozoa; *Anopheles dirus*; Diptera; Haemolymph; Haemocyte; Melanization; Prophenoloxidase (PPO); Phenoloxidase (PO); Monophenol oxidase (MPO); *o*-Diphenoloxidase (*o*-DPO); Refractory

1. Introduction

Malaria, one of the most devastating diseases in the world, is caused by genus *Plasmodium. Plasmodium* requires completely development in the mosquito to be transmitted to a new vertebrate host. However, mosquito can limit or even entirely block the development of *Plasmodium.* For example, a lot of *Plasmodium* are killed due to the absence of mosquito-derived molecules essential for development or the presence of PM (peritrophic matrix) (Billingsley and Rudin, 1992) and anti-malarial trypsin-like activity of proteases within the mosquito midgut (Gass and Yeates, 1979; Muller et al., 1993) before ookinetes transverse the midgut epithelium. Once ookinetes begin to transverse the midgut epithelium, robust local and systemic innate immune responses, including induction of defensin (Lowenberger et al., 1999) and NOS (nitro oxide synthease) (Luckhart et al., 1998), are initiated to largely limit the development of *plasmodium.* Most importantly, the development of a wide variety of *Plasmodium* can be fully blocked by melanotic encapsulation of late ookinetes or early oocysts in a genetically selected refractory mosquitoes, *An. gambiae* (*Anopheles gambaie*) L35 (Collins et al., 1986; Paskewitz et al., 1988). It implied that melanotic encapsulation is the key defense mechanism of the refractory *Anopheles* against *Plasmodium.*

Melanotic encapsulation, also called melanization, is characterized as depositing of melanin on the invading pathogen surface. It is known that melanin synthesis in mosquito and other insects involves several enzymes, including PAH (phenylalanine hydroxylase), PO (phenoloxidase) and DCE (dopachrome conversion enzyme)

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(Zhao et al., 1995). Although both PAH (Infanger et al., 2004) and DCE (Han et al., 2002) can accelerate and enhance melanization reaction of insect against invader, PO is regarded as the key enzyme in generation of melanin (Christensen et al., 2005). PO has two activities: MPO (monophenoloxidase) that hydroxylates tyrosine to a dihydroxylated product and DPO (diphenoloxidase) that oxidizes the dihydroxylated product to a quinone and leads to melanin formation. As a result, melanin, together with cytotoxic intermediates, isolated and killed the invaded pathogens. Knock-down experiments by transducing mosquitoes with anti-sense RNA that targeted to the copper-binding region of PO almost entirely abolished the melanization of microfilariae, demonstrating a pivotal role of PO in melanization reaction (Shiao et al., 2001; Tamang et al., 2004).

POs, however, always exist as proenzyme, prophenoloxidase (PPO), that are produced by haemocytes and released into the haemolymph (Durrant et al., 1993). Genomic analysis revealed that *An. gambiae* contained nine putative PO-coding sequences (Christophides et al., 2002). Although PPO2, PPO3 and PPO9 were induced following blood feeding (H. M. Muller, unpublished data), no PPO was upregulated upon infection of *Plasmodium* (Muller et al., 1999). However, emergent evidence did support the important role of PO in melanization of *Plasmodium* by *Anopheles*. Two-dimensional gel analysis of haemolymph proteins from *Plasmodium*-melanizing and -non-melanizing strains of *An. gambiae* identified a refractory strain-specific polypeptide AgSp14D1 (Chun et al., 2000) that was mapped together with *Pen*3, a minor quantitative trait loci of melanizing phenotype of the refractory strain, *An. gambaie* L35 (Zheng et al., 1997). Phylogentic analysis grouped the AgSp14D1 with three PPAEs (prophenoloxidase-activation enzymes) that function in PPO activation. And existing of a MPF (melanization preventing factor), a putative serine protease inhibitor, in susceptible strain haemolymph (Paskewitz and Riehle, 1998) further suggested the important role of PO in melanotic encapsulation of *Plasmodium* by *An. gambiae*.

An. dirus (*Anopheles dirus*), the main Southeast malaria vector, is known to be susceptible to human malaria parasites, but naturally refractory to rodent malaria parasite, *Plasmodium yoelii* (*P. yoelii*) (Somboon et al., 1999). However, the knowledge of mechanism of *An. dirus* naturally refractory to *P. yoelii* is still undefined. Here, we found that some *P. yoelii* taken into *An. dirus* could develop into oocyst, but oocysts were partially melanized at 7 days and wholly melanized at 15 days post-infective blood meal, suggesting that melanization of most oocysts was one of the immune responses of *An. dirus* against *P. yoelii*. In addition, activity of PO, such as MPO and *o*-DPO, in haemolymph of *An. dirus* fed on infectious blood meal was significantly increased when compared to those fed on 5% glucose or normal mouse blood ($p < 0.05$), indicating that PO might be involved in this process.

2. Materials and methods

2.1. Mosquitoes rearing and infection of P. yoelii

Anopheles dirus (Hainan strain) are routinely maintained in cage at 27–28 °C, 70% humidity and fed on 5% glucose. For female adults to lay eggs, we fed the mosquitoes with mouse blood after a 3 h starvation. Larvae were kept in water and fed on yeast powder, and pupa emergences in the cage.

For infection experiment, female adults (3–5 days old) were first fed on mouse blood containing activated gametocytes of *P .yoelii* (BY265 strain) after a 3 h starvation, then were fed on 5% glucose.

2.2. Transmission electronic microscopy

Infected guts were fixed in a fixative (2% glutaraldehyde in 0.05 M Na–phosphate, pH 7.6) at 24, 48, 72, 96 and 120 h after the blood meal. After an 8–12 h fixation at 4 °C, midguts were washed in 0.05 M Na–phosphate and dehydrated in a series of increasingly concentrated ethyl alcohol dilutions, and infiltrated with a mixture of propylene oxide and Epon-Araldite, and embedded in Epon-Araldite. Thick sections (0.5–1 μM) were cut with glass knives and stained with toluidine blue. When capsules were observed with light microscopy, thin sections cut on a Sorvall MT-2B ultramicrotome Grids were stained with lead citrate followed by uranyl acetate and examined with a Philips 400 electron microscope. At least 30 midguts were sectioned for each time period.

2.3. Detection of PO activity within gels

Haemolymph collected by centrifugation procedure (Sidjanski et al., 1997) was used to native PAGE electrophroesis separation and proceed to PO activity detection at 5, 7, 11 and 15 days post-infection, respectively.

The MPO activity was assayed by incubation of the gel in 10 ml of 0.067 M sodium phosphate buffer (pH 6.9, 0.01 M tyrosine methyl ester, 2.5 mg pheazine methosulfate, 5 mg nitro blue tetrazolium) for 45 min in the dark at room temperature. To detect *o*-DPO activity, gel was incubated in 0.05 M sodium phosphate buffer consisted of 8 ml of 0.01 M dopamine (pH 6.8) and 2 ml 0.3% 3-methyl-2-benzothiozolinone (MBTH) in ethanol for 30–45 min in the dark at room temperature. PO inhibitors, such PTU (phenylthiourea) and tropolone, were added to determine the specific activity of both MPO and *o*-DPO, and authentic bands were scanned and read out as OD value.

2.4. SDS–PAGE

Haemolymph collected by expulsion method (Chun et al., 2000) was proceeded to SDS–PAGE. In brief, haemolymph was expelled in a droplet at the tip of the proboscis upon pressure to the thorax, following the tip of the proboscis was removed with microscissors from