INSTITUT PASTEUR

Microbes and Infection 9 (2007) 192–199

www.elsevier.com/locate/micinf

Original article

# Laminin and a *Plasmodium* ookinete surface protein inhibit melanotic encapsulation of Sephadex beads in the hemocoel of mosquitoes

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Received 31 August 2006; accepted 7 November 2006
Available online 8 December 2006

**Abstract**

In refractory mosquitoes, melanotic encapsulation of *Plasmodium* ookinetes and oocysts is a commonly observed immune response. However, in susceptible mosquitoes, *Plasmodium* oocysts develop extracellularly in the body cavity without being recognized by the immune system. Like *Plasmodium gallinaceum* oocysts, negatively charged carboxymethyl (CM)-Sephadex beads implanted in the hemocoel of *Aedes aegypti* female mosquitoes were not usually melanized, but were coated with mosquito-derived laminin. Conversely, electrically neutral G-Sephadex beads were routinely melanized. Since mosquito laminin coated both CM-Sephadex beads and *P. gallinaceum* oocysts, we hypothesized that laminin prevents melanization of both. To test this hypothesis, we coated cyanogen-bromide-activated G-Sephadex beads with laminin, recombinant *P. gallinaceum* ookinete surface protein (*Pg*S28) or bovine serum albumin (BSA). Beads were implanted into the abdominal body cavity of female *Aedes aegypti* and retrieved 4 days later. Uncoated controls as well as BSA-coated G-Sephadex beads were melanized in a normal manner. However, melanization of beads coated with mouse laminin, *Drosophila* L2-secreted proteins or *Pg*S28 was markedly reduced. Fluorescent antibody labeling showed that *Pg*S28-coated beads had adsorbed mosquito laminin on their surface. Thus, mosquito laminin interacting with *Plasmodium* surface proteins probably masks oocysts from the mosquito's immune system, thereby facilitating their development in the body cavity.


*Keywords:* *Plasmodium*; Oocyst; *Aedes aegypti*; Basement membrane; Laminin

## 1. Introduction

Sporogony in malaria parasites begins with gametogenesis, fertilization and ookinete maturation within the mosquito's blood meal. Motile ookinetes traverse the midgut epithelium and, upon contact with the basement membrane (BM), metamorphose into spherical oocysts that develop extracellularly [1]. Recognition of mosquito BM and adhesion to it are crucial stages in the sporogonic cycle of *Plasmodium,* and laminin, a major component of the BM, has been implicated in this interaction [2–6]. Although oocysts can be cultured in vitro in the absence of laminin [7], efficient oocyst growth and maturation are dependant on mosquito-derived laminin, which is a key component of the oocyst capsule [2].

Invertebrates lack adaptive immunity and rely on innate immune responses to fend off pathogens. Innate immunity is based upon receptor-mediated recognition of invading organisms that triggers synthesis of potent antimicrobial peptides, phagocytosis and encapsulation [8]. Encapsulation in the hemocoel of insects is frequently manifested by the production of melanin, which is deposited on the surface of foreign bodies to form a rigid melanotic capsule [9–11]. Melanization responses are not uniform and depend on the chemical composition and electric charge of implanted foreign bodies [12–14]. In mosquitoes, glucan-based Sephadex beads made of cross-linked dextran were routinely melanized, while beads made of other types of matrices were not [13]. These findings make sense, since β-1,3-glucans are recognized as potent

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doi:10.1016/j.micinf.2006.11.006

immune elicitors and a β-1,3-glucan-specific pattern recognition receptor that promotes melanization was identified in the mosquito *Armigeres subalbatus* [15,16].

The capacity of refractory strains of *Anopheles gambiae* to melanize *Plasmodium* oocysts and Sephadex beads was shown to be governed by a shared genetic mechanism [17,18]. Thus, Sephadex beads are legitimate surrogates for oocysts and potentially useful tools for studying mosquito reactions to infection. Using the implanted Sephadex-bead model, we were able to demonstrate that Sephadex beads are invariably encapsulated. The more obvious melanotic capsule frequently encapsulates G-Sephadex. A laminin-based translucent proteinaceous coat is deposited on negatively-charged CM-Sephadex beads, preventing their melanization.

## 2. Materials and methods

### 2.1. Mosquitoes

*Aedes aegypti* mosquitoes (Liverpool Black Eye strain) were reared at 26 ± 1 °C and 70 ± 10% relative humidity. For injections of beads, 4–7-day-old females were lightly anesthetized using $CO_2$ and placed in a Petri dish over wet ice. Each female was injected through the membranous cuticle connecting abdominal segments with pre-wetted (24 h) Sephadex beads (20–80 μm diameter, Farmacia) suspended in approximately 2 μl *Aedes* saline [19] using pulled glass microcapillaries. Injection into the abdomen rather than the thorax allowed us to implant 20–30 beads into each female, placing them close to the midgut where *Plasmodium* oocysts develop, and facilitating their subsequent retrieval for analysis. Females were transferred to a holding cage kept at high relative humidity and provided with 30% sugar solution. To determine levels of melanization 4 days post-injection, mosquitoes were anesthetized using $CO_2$ and placed in a Petri dish over wet ice. Guts were gently pulled out and the abdominal wall was broken open using watchmakers' forceps. The entire preparation was examined at ×200 magnification under differential interference contrast illumination. Beads that had spilled out were counted and scored for melanization. The percent melanized surface area was approximated and each bead was assigned to one of three categories (no melanization; 1–49% melanization; or 50–100% melanization, Fig. 5, bottom panel).

### 2.2. Cell culture

Schneider's L2 *Drosophila* cells are known to secrete *Drosophila* laminin and support in vitro culture of *Plasmodium* oocysts [20–22]. L2 cells were adapted to and cultured in serum-free medium (*Drosophila* SFM: Gibco-BRL) at 26 °C to a concentration of $1.5\times10^7$ cells/ml. Cultures were centrifuged at 2000 × *g* for 10 min and the supernatant fluid was collected and supplemented with a protein inhibitor cocktail (final concentrations: 10 mM EDTA, 40 μg/ml AEBSF, 7 μg/ml leupeptin, 1 mM iodoacetamide, 2 mM phenanthroline [Sigma]). Protein from spent culture overlays was concentrated to ~6 mg/ml by centrifugation through 10 kD cutoff membranes (Centricon Centrifugal filter units, Millipore).

### 2.3. Activation and protein coating of G-Sephadex beads

950 mg wet weight of G-Sephadex beads were suspended in 1.9 ml, 50% acetone on ice. 1 M cyanogen bromide (CNBr) dissolved in190 μl acetone was added and mixed with the bead suspension on ice. Aqueous triethylamine (TEA) (190 μl/1.5 M) was dripped slowly into the slurry and mixed vigorously. Beads were washed several times in cold 50% acetone and several times in cold water. Proteins from spent L2 cell culture media, murine laminin or BSA (Sigma), 6 mg/ml in PBS were mixed with equal volumes of 0.2 M sodium acetate ($CH3COONa \cdot 3H_2O$ pH 4.0). Recombinant *Plasmodium gallinaceum* surface protein 28 (*Pg*S28) was obtained from the Malaria Reference and Resource Center (MR4) [23]. *Pg*S28 (0.5 mg/ml) was mixed with equal volumes of $NaHCO_3$ (0.2 M, pH 8.6). The mixture was stirred gently overnight at 4 °C. Beads were washed several times with PBS, blocked with 0.5 M ethanol amine and washed again. Protein concentrations were determined before and after coupling to verify adsorption by the activated beads, which was 60–70%.

### 2.4. Parasites

*P. gallinaceum* (8A strain) was maintained in 2–5 week-old chickens by weekly serial blood passage from infected donor chickens. Mosquitoes were infected by feeding directly on an anesthetized chick with rising parasitemia of 10–20%.

### 2.5. Antibodies

Anti-murine laminin was produced in rabbits using Engleberth Holm Swarm tumor-derived laminin (Sigma). The α-bead serum was raised in mice inoculated with 500 non-melanized CM-Sephadex beads recovered from *A. aegypti* females after 3 days using RIBI adjuvant. Mice were subsequently boosted twice on days 21 and 42 post-injection. The anti-*P. gallinaceum* ookinete surface protein 28 kDa (α-Pgs28) mAb designated B3B3 was a gift from D. Kaslow.

### 2.6. Western blots and indirect immunofluorescence

Protein from spent L2 cell culture media was concentrated to 6 mg/ml, electrophoresed on standard 4–20% gradient polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked using 5% skimmed milk and incubated with α-laminin serum at 1:100 dilution for 1 h. Following washes and blocking steps, blots were incubated with α-rabbit peroxidase and developed using substrate for enhanced chemilumnescence.

Fluorescent labeling of beads was performed as follows: 1 h blocking in 5% skimmed milk, incubation of beads in a microtube with 1:100 dilution of the primary antibody, 3 washes in PBS (0.5% tween 20), incubation with α-rabbit or α-mouse